CHARACTERIZATION OF *BER* (*Zizyphus. spp.*) BY DNA FINGERPRINTING

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in
Biotechnology

By

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Certificate

This is to certify that the work entitled **"Characterization of *Ber* (*zizyphus. spp.*) by DNA Fingerprinting"** is a piece of research work done by **Ms. Devanshi** under our guidance and supervision for the degree of Doctor of Philosophy in Biotechnology at Department of Biotechnology, J.C. Bose Institute of Life Sciences, Bundelkhand University, Jhansi (U.P.) India. The candidate has put-in an attendance of more than 200 days with us.

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3. fulfills the requirements of the ordinance relating to the Ph.D degree of the University; and
4. is upto the standard both in respect of contents and language for being referred to the examiner.

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Declaration

I, Devanshi, hereby declare that the thesis entitled **"Characterization of *ber* (*zizyphus. spp.*) by DNA Fingerprinting"** is my own work conducted under the supervision of Dr. **Bhanumati Singh** and Co-supervision of **Dr. A.K. Singh** at Department of Biotechnology, J.C. Bose Institute of Life Science, Bundelkhand University, Jhansi, approved by Research Degree Committee.

I further declare that to best of my knowledge the thesis does not contain any work, which has been submitted for the award of any degree either in this university or in any other university/deemed university without proper citation.

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Dedicated to my Parents

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrary Primed PCR
bp	Base pair
ASAP	Arbitrary Signature from Amplification Profile
CAPS	Cleaved Amplified Polymorphic Sequence
cAFLP	Chemiluminiscent Amplified Fragment Length Polymorphism
c-DNA	Complementary DNA
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribose Nucleic Acid
DAF	DNA Amplification Fingerprinting
°C	Degree Celsius
EMR	Effective Multiplex Ratio
EtBr	Ethidium Bromide
EST	Expressed Sequence Tags
GOT	Glutamate Oxaloacetate Transaminase
GD	Genetic Distance
g	Grams
ISSR	Inter Simple Sequence Repeats
Ib	Band Informativeness
LB	Leuria Broth
LD	Linkage Disequilibrium

MI	Marker Index
Min	Minutes
MLG	Multilocus Genotype
$MgCl_2$	Magnisium Chloride
MP-PCR	Microsatellite Primed Polymerase Chain Reaction
µM	Micro molar
µl	Micro litre
mM	Mili molar
PIC	Polymorphism Information Content
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAHM	Random Amplified Hybridization Microsatellite
RAMP	Random Amplified Microsatellite Ppolymorphism
RAMPO	Random Amplified Microsatellite Polymorphism
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rRFLP	Radioactivity Restriction Fragment Length Polymorphism
Rp	Resolving power
SCAR	Sequence Characterized Amplified Region
SDS	Sodium Dodecyl Sulphate
SFRA	Selective Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism

SSC	Sodium Citrate Saline
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeats
SSRIT	Simple Sequence Repeat Identification Tool
STMS	Sequence Tagged Microsatellite
STS	Sequence Tagged Sites
UPGMA	Unweighted Pair Group Mean Cluster Analysis
VNTR	Variable Nucleotide Tendom Repeats
X-gal	5-Bromo-4-chloro-3-indolyl-D-galactopyraniside

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Chapter 1

INTRODUCTION

The genus *Ziziphus* commonly known as *ber* (jujube) belongs to the buckthorn family Rhamnaceae, which comprised of 50 genera and more than 600 species. The generic name *Ziziphus* has been derived from "Zizouf", an ancient Greek name derived from the Persian word zizafun. Paclt (1999) advocates use of *Zizyphus* (instead of *Ziziphus*), this is contrary to earlier belief that *Ziziphus* is the correct spelling (Hayes, 1957). It is a genus of about 40 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the Northern hemisphere (Ray, 2002), of which *Z. jujube* Miller and *Z. mauritiana* Lamarck are the most important in terms of distribution and economic significance. South and Southeast Asia is the centre of both evolution and distribution of the genus *Ziziphus* (Liu and Cheng, 1995). *Ziziphus mauritiana* Lam. is classified as Indian jujbe (*ber* in hindi) and is cultivated all over the arid parts of Indian subcontinent. *Ziziphus* plant develops a deep root system that ensures its ability to exploit deep water sources, thereby maintaining a sufficient water and nutrient supply for prolonged dry periods (Depommier, 1988). Different species of *Ziziphus* can tolerate a very low temperature and therefore is suitable for growing in colder regions like China, Spain, France, etc. Since *Ziziphus* trees can tolerate excessive stress from drought, salinity and even water logging in the soil, they are considered appropriate for planting on corrupted and subsidiary lands (Depommier, 1990). Several physiological and morphological characteristics are responsible for their ability to survive in arid and semi arid environments. The tree is endowed with a high degree of edaphic and climatic adaptability and can grow in different kind of soil types from neutral or slightly alkaline to moderately saline soils and also in deep, sandy loams including the sub marginal lands

between latitudes of 34° S and 51° N, up to 2,800 m above sea level. *Ziziphus* species are potentially very important in preventing soil erosion and desertification and has therefore been planted in India and several other countries (Pareek *et al.,* 2001). The wide geographical and climatic distribution is indicative of the fact that there exists a tremendous genetic diversity in *ber* that needs to be identified and catalogued. However, indiscriminate felling of *ber* trees in the last 50 years has caused serious state of deterioration of their genetic resources. It is a multipurpose tree that provides fruit, fodder and fuel (Vashishtha, 1997) and biofencing (Jones *et al.*, 1998). The *ber* tree is suitable to rehabilitate extensive, resource poor areas and also exists in wild groves that are found in the warmer parts of India, Pakistan, Bangladesh, Sri Lanka, etc.

Z. mauritiana, the cultivated species in India can be traced back to as early as 1,000BC and can be ideally described as-"a gift of mother nature" which symbolizes the productive capacity of the seemingly infertile ecosystem. The estimated area in India under regular plantations of improved varieties is about 70,000 ha (Pareek, 1996). In India, the scions of varieties which have been selected to improve the yield and fruit quality are routinely grafted on to the vigorous rootstocks of wild species to provide a reasonable cash crop on land which is unsuitable for other forms of cultivation (Cherry, 1985). Under ideal environmental conditions, Z. mauritiana exhibits very high rates of net photosynthesis and stomatal conductance. Surplus carbohydrates are assimilated as starch in the roots if not invested in growth, leading to very high reserves of carbohydrate in the belowground structures. The fruits of Indian *ber* have high contents of protein, minerals and vitamins A and C than apples and citrus (Khoshoo *et al.,* 1985) and contain

20 to 30% sugar, up to 2.5% protein and 12.8% carbohydrates. Because of high nutritional value it has a great commercial importance. The drupes are eaten fresh, pickled, dried or made into confectionery; the juice can be made into a refreshing drink (Coates, 1990), jam and *kachaso*, a crudely distilled spirit of considerable potency (Perera *et al.,* 1998). For farmers, *Z. mauritiana* is of interest because it is fast growing bearing fruits within 3-4 years (Khoshoo *et al.,* 1985). The large carbohydrate reserves in the roots contribute to the strong regeneration potential of *Ziziphus* plants. *Z. mauritiana* has a great power to recover from injury including fire, and thrives on burnt grassy tracts (Grice 1996, 1997). The major *ber* growing states are Haryana, Punjab, Uttar Pradesh, Rajasthan, Gujarat, Madhya Pradesh, Bihar, Maharashtra, Andhra Pradesh and Tamil Nadu.

Ziziphus plants are cross-pollinated and therefore, the natural population, which largely regenerates through seeds, exhibits a vast range of genetic heterogeneity. The commercial varieties of *ber* have been evolved through selection of promising types from this wide spread natural variability followed by budding on suitable rootstocks. In evolutionary terms, this genetic variability may well benefit *Ziziphus* in harsh environments by allowing rapid adaptation to changing environmental conditions at a population level. The chromosome num*ber* for the genus has been reported to be n =20 (Srinivasachar, 1940), n = 36, 48 (Srinivasan, 1952) and n = 24, 48 (Khoshoo and Singh, 1963, Wang *et al.*, 1998) for the Indian jujube. Khoshoo and Singh (1963) have reported that the genus *Ziziphus* is monobasic with x = 12 and not a tribasic (x=10, 11, 12) as assumed by Darlington and Wylie (1955).

At the fruit orchard of Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi (India) a large collection of *ber* has been done to safeguard the genetic resources of this species. A single variety of *ber* sometimes is known by local acronyms in various parts of the country. Assessment of genetic diversity has traditionally been through morphological characters and biochemical markers (Oh *et al.,* 1988), which has often found to be less effective. Moreover, genetic analysis and breeding of woody perennial like *ber* is complicated by many factors including long periods of juvenility (up to 7 years), high ploidy levels, lack of described Mendelian marker and so on (Rowland and Levi, 1994). To establish the correct identity of genotypes molecular definition of genotypes is imperative. The application of molecular markers in plant improvement has several distinct advantages over the traditional phenotypic markers available to plant breeders, the most important being uninfluenced by the environmental conditions. Molecular markers based on polymerase chain reaction (PCR) method offer several advantages over the sole use of conventional morphological markers. Over the last fifteen years PCR technology has led to the development of two simple and quick techniques called RAPD and ISSR. The former detects nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence (Williams *et al.,* 1990) and the latter permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Wu *et al.,* 1994 and Zietkiewicz *et al.,* 1994). Although genetic diversity analysis and cultivar identification by RAPD and ISSR markers have been performed in many fruits (Cervera, 1998 and Ricciardi *et al.,* 2002), its application for

Ziziphus species and its relatives has not been carried out (Weekly *et al.*, 2002). The RAPD and ISSR markers are beginning markers for plant species but both the markers lack reproducibility and are of dominant nature. Therefore, with the new advances in molecular markers the need is to develop highly polymorphic and reproducible, codominant molecular markers that are distributed throughout the genome *e.g.* markers based on microsatellite sequences (SSR markers). The present investigation is an effort to characterize a collection of *ber* genotype and to establish genetic relatedness using molecular marker.

The present investigation has been undertaken with the following objectives:

1. To develop DNA profile using RAPD marker for 48 varieties/genotypes
2. To develop DNA profiles using ISSR marker
3. To develop new SSR marker and its use
4. Data analysis and interpretation of results
5. Development of DNA fingerprinting combining all the marker (RAPD, ISSR and Microsatellite) used.

Chapter 2

REVIEW OF LITERATURE

The genus, *Ziziphus* (jujube), a member of Rhamnaceae is comprised of approximately 170 species of deciduous/ evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (Liu and Cheng, 1995) between 34° S and 51° N latitude, and up to 2800 m above sea level in different continents (Table 1) with a few species occurring in temperate regions. The number of species reported in the genus *Ziziphus* is variable: 18 species and 5 intermediate species (Hooker, 1875), 50 species (Watt, 1893), 60 species (Ridley, 1922), 40 species (Rendley, 1959), 80 species (Evreinoff, 1964), 86 species (Johnston, 1972), 135 species of which 90 are found in the old world and 45 in the new world (Bhansali, 1975) and 170 species (Liu and Chang, 1995). Out of the total species in the genus *Ziziphus* 88 (51.8%) are concentrated in Asia, followed by North America (31), South America (28), Africa (28), Oceana (9) and Europe (5). However, the most distinctive species are found in North and South America (28 and 27, respectively) (Liu and Cheng, 1995). Some species, like *Z. mauritiana* and *Z. jujuba*, occur nearly in every continent, whereas other species, like *Z. nummularia*, *Z. spina-christi* and *Z. mucronata* are restricted to distinct areas (Table 2.1). *Ziziphus* species can grow either as trees and shrubs (*Z. mauritiana, Z. rotundifolia, Z. jujuba, Z. mucronata*) or exclusively as small shrubs or bushes (*Z. nummularia, Z. lotus, Z. spina-christi, Z. obtusifolia*).

In both China and India, *Ziziphus* trees have a long tradition of selection and cultivation, with the result that the species occurring in these countries (*Z. mauritiana, Z. jujuba*) are better known and more widely researched than those in other regions. These two species are economically important and being cultivated for their fruit (Islam and Simmons, 2006). *Ziziphus mauritiana* Lam, also known as *ber*/Indian jujube/ Indian

plum/ Chinee Apple / Desert apple is an evergreen, medium-sized, thorny tree with ability to thrive and produce fruits in arid or semi-arid regions. It is native from the Province of Yunnan in southern China to Afghanistan, Malaysia and Queensland, Australia. It is cultivated to some extent throughout its natural range but mostly in India where it is grown commercially and has received much horticultural attention and refinement despite the fact that it frequently escapes and becomes a pest.

Table 1: Worldwide Distribution of *Ziziphus* Species

Continent	*Zizihphus species*	Part of continent
Africa	*Z. abyssinica* Hochst.	Tropical Africa
	Z. lotus Lamk	Northern Africa
	Z. mauritiana Lamk.	Tropical Africa, Sahel Zone, Zimbabwe
	Z. mucronata Willd.	Southern Africa
	Z. spina-christi Willd	Middle east
Asia	*Z. jujuba* Mill.	China, India, Korea, Malaysia
	Z. mauritiana Lamk.	China, India, Pakistan, Malaysia
	Z. nummularia W.i.A.	India
	Z. oenoplia Mill.	Tropical Asia
	Z. rotundifolia Lam.	India
	Z. rugosa Lam.	India
	Z. sativa Gaertn.	Pakistan
	Z. spina-christi Willd.	Middle east
	Z. xylopyra Willd	India
Australia	*Z. mauritiana* Lamk	
Europe	*Z. jujuba* Mill.	Mediterranean
	Z. lotus Lamk.	Mediterranean
	Z. mauritiana Lamk.	Mediterranean
	Z. sativa Gaertn.	Mediterranean
North America	*Z. amole* M.C.Johnst.	Mexico
	Z. celata J.i.H.	USA
	Z. jujuba Mill.	USA
	Z. mexicana Rose	Mexico
	Z. obtusifolia Gray	Mexico, USA
South America	*Z. cinnamomeum* Tr.&Pl.	Venezuela
	Z. mistol Griseb.	Argentinia, Paraguay
	Z. joazeiro Mart.	
	Z. oblongifolia S.Moore	

The tree does best on sandy loam, neutral or slightly alkaline. It also grows well on laterite, medium black soils with good drainage, or sandy, gravelly, alluvial soil of dry riverbeds where it is vigorously spontaneous. These trees perform well even on marginal and inferior lands where most other fruit tree species either fail to grow or give poor performance (Jawanda and Bal, 1978). In India during summer months of May and June, *Z. mauritiana* enters into dormancy by shedding its leaves. The scions of varieties which have been selected to improve the yield and fruit quality are routinely grafted on to the vigorous rootstocks of wild species to provide a reasonable cash crop on land which is unsuitable for other forms of cultivation (Cherry, 1985). *Ziziphus* species have several physiological and morphological characteristics that may contribute to their ability to adapt to arid environments and make it important for the integrated economy of the arid lands. Species of fruit trees in the genus *Ziziphus* represent examples of such multipurpose plants with great potential for selection and use in drought-prone regions. *Z. mauritiana* is an example of an extremely drought-hardy species, and is a dominant component of the natural vegetation of the Indian desert (Cherry, 1985). These trees are well adapted to seasonal drought and hot conditions. They play an important role in the soil erosion with their strong root system that ensures its ability to exploit deep water sources, thereby maintaining a sufficient water and nutrient supply for prolonged periods when the upper soil layers are drying out (Singh *et al.*, 1998) and assimilation/leaf conductance ratio than in unstressed trees (Jones, 1992). An indication of the importance of the root is the high root-to-shoot ratio of *Z. mauritiana* and deep rooting which has been reported as a characteristic of both *Z. nummularia* and *Z. mauritiana* (Depommier,

1988). The leaves of *Ziziphus* provide fodder for livestock; the hard wood is used for making agricultural implements, fuel and high quality charcoal. In many regions, *Ziziphus* is grown as a hedge, with its spines creating effective live fencing. Its fruits are highly nutritious and provide a valuable source of energy, vitamins. In addition, extracts from fruits, seeds, leaves, roots and bark of the trees are used in many traditional medicines to alleviate the effects of insomnia, skin diseases, inflammatory conditions and fever. Juice of the root bark is said to alleviate gout and rheumatism and an infusion of the flowers serves as an eye lotion. *Ber* trees appear to tolerate roadside pollution because they maintain high chlorophyll content, particularly chlorophyll b, although their growth becomes retarded (Hussain *et al.,* 1994). Plants affected by fire damage regenerate in short time. The regrowth is mainly from new shoots that arise from just above or just below ground level in the case of young plants and from the buds in the canopy or epicormic buds on the stems of older plants (Grice and Brown, 1996; Grice, 1997).

At about the beginning of the Christian era, the Chinese jujube was imported into Europe and is now widely distributed throughout Persia, Armenia, Syria and the Mediterranean regions in Spain and France. Reports of early writers and explorers emphasized the heat and drought tolerance of jujubes, and probably because of this, jujubes were thought most likely to succeed in the dry regions of the southwestern US (Locke, 1947). *Z. mauritaina* was introduced into Guam about 1850 but is not often planted there or in Hawaii except as an ornamental. Specimens are scattered about the drier parts of the West Indies, the Bahamas, Colombia and Venezuela, Guatemala, Belize, and southern Florida. In Barbados, Jamaica and Puerto Rico the tree is naturalized

and forms thickets in uncultivated areas. In 1939, six trees from Malaysia were introduced into Israel and flourished there. Some improved Indian cultivars like Gola and Seb have been imported to Israel and Africa and are the grafts onto native rootstocks of *Z. spina-christi* and *Z. abyssinica*, respectively (Cherfas, 1989). The same technique was successfully used in Zimbabwe to produce high-quality Indian selections on the native *Z. nummularia* rootstock species (Kadzere and Jackson, 1997).

The fruit of *ber* is known as drupe. The fleshy drupes of several species are rich in sugars and vitamins, and this fact has made. The skin of fruit most is smooth and greenish-yellow to yellow. The drupes are eaten fresh, pickled, dried or made into confectionery, and the juice can be made into a refreshing drink (Khoshoo and Subrahmanyam, 1985). In Zimbabwe, *Ziziphus* fruits are used to produce jam and kachaso, a crudely distilled spirit of consirable potency (Coates Palgrave, 1990). Though the fruit does not find much favour with the upper classes (poor man's fruit, Khoshoo and Subrahmanyam, 1985), it has a high nutritional value and a great commercial potential. Consequently, in many regions of the world, *Ziziphus* fruits are sold on local markets, generating cash income for people of rural areas and improving family nutrition. Drupes have higher contents of protein and vitamins A and C than apples (Anonymous 1976). The fruits contain between 70 and 165 mg ascorbic acid per 100 g of pulp, which is two to four times higher than the vitamin C content of citrus fruits. The mineral content of calcium, phosphorus and iron in *Z. mauritiana* fruits is also reported as being higher than in apples and even oranges (Jawanda and Bal, 1978). For farmers, *Z. mauritiana* is an interesting crop because it is so fast growing and bears fruits within 2-3 years (Jawanda

and Bal, 1978). Jujubes (*Z. jujuba*) were eaten by the ancients of the chalcolitic age (1500-1000 b.c.) and the fruits have been in cultivation for the past 400 years in both India and China (Anonymous, 1976).

The food value per 100 gm of edible portion of fresh fruit includes: moisture (81.6-83.0 g), protein (0.8 g), fat (0.07 g), fiber (0.60 g), carbohydrates (17.0 g), total sugars (5.4-10.5 g), reducing sugar (1.4-6.2 g), non-reducing sugar (3.2-8.0 g), ash (0.3-0.59g), , calcium (25.6 mg), phosphorous (26.8 mg), Iron (0.76-1.8 mg), carotene (0.021 mg), thiamine (0.02-0.024 mg), riboflavin (0.02-0.038 mg), niacin (0.7-0.873 mg), citric acid (0.2-1.1 mg), ascorbic acid (65.8-76.0 mg), fluoride (0.1-0.2 ppm) and pectin (2.2-3.4%) .

Under ideal environmental conditions, *Z. mauritiana* exhibits very high rates of net photosynthesis and stomatal conductance, high rate of nitrate reductase activity (NRA) in leaves of *Z. mauritiana* (1 μmol NO_2- g FM^{-1}- h^{-1}) measured in leaves of drought-stressed plants. Consequently, total nitrogen content of the leaves is very high, which is a mesic character of *Ziziphus* leaves, that lack xeromorphic adaptations such as heavy cuticularization, or deep folds in their surfaces with sunken stomata. The combination of high levels of NRA and net photosynthesis results in a high relative growth rate, essential if these plants are to compete effectively during brief periods of active growth. The large carbohydrate reserves in the roots contribute to the strong regeneration potential of *Ziziphus* plants. *Z. mauritiana* is reported as having a great power to recover from injury of any kind, including fire, and thrives on burnt grassy tracts (Anonymous, 1976; Grice, 1996). After such events, most plants of Z. *mauritiana*

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resprouted vigorously within 3 months and by the 4th month after fire, burnt and unburnt plants were similar with respect to the distribution of individuals and their physiological characteristics (Grice, 1997). Because of its ability to resprout from both crown and roots, along with its resistance to herbicides, *Z. obtusifolia* has demonstrated its ability to increase its cover on Texas rangeland after the release of competition from other woody vegetation by bush control treatments (Speer and Wright, 1981). There are reports that wild jujube plants (*Z. lotus*) also have the ability to resprout vigorously even after being cut to ground level in the previous fall in Morocco (Regehr and El Brahli, 1995). *Ziziphus* plants are cross-pollinated and are highly out breeding, and as a result of this, the natural population, which largely regenerates through seeds, exhibits a vast range of genetic heterogeneity. The potential of this variability has been severely underutilized. In contrast, the commercial cultivars, which are clonally propagated via budding on a suitable rootstock, have retained their genetic fidelity. In evolutionary terms, this genetic variability may well benefit *Ziziphus* in harsh environments by allowing rapid adaptation to changing environmental conditions at a population level.

In India, more than 90 cultivars differing in the habit of the tree, leaf shape, fruit form, size, color, flavor, keeping quality, and fruiting season have been reported. Among the important cultivars, eleven are described in the encyclopaedic *Wealth of India:* 'Banarasi (or Banarsi) Pewandi', 'Dandan', 'Kaithli' ('Patham'), 'Mundia Mahrara', 'Narikelee', 'Nazuk', 'Sanauri 1', 'Sanauri 5', 'Thornless' and 'Umran' ('Umri'). *ber* cultivation has not assumed large proportion due to several constraints. *ber* orchards are

located mainly in ecologically deprived, stress prone areas where production, marketing and managerial skills are poorly developed. The farmers face following constraints:

A) Unavailable information on (i) Modern production techniques, and (ii) Post harvest management and marketing

B) Lack of inputs for (i) reliable planting material of recommended variety, and (ii) reliable plant protection equipments and chemicals

C) Absence of infrastructures for (i) regulatory mechanism to ensure supply of good quality and reliable planting material, and (ii) checks to regulate supply of good quality plant protection chemicals.

Research initiatives on *ber* in India until the 1960 were limited to varietal trials and vegetative propagation studied in the states of Punjab and Uttarpradesh. Major research initiatives took place during the last four decades of the twentieth century.

2.2 Molecular Markers and Their Use in Plants

Plant genetic resources are one of the most valuable assets available to mankind. Protection, preservation and conservation of these resources for posterity, therefore, assume enormous significance. An important component for effective and efficient management of plant genetic resources as well as their utilization is characterization of the germplasm. Such a characterization is essential not only for identification of various species but also to determine their genetic relatedness. The information generated could be used successfully in breeding programmes wherever possible. This also assumes great relevance in the present context of intellectual property rights and trade agreements.

Markers for identifying and manipulating genes have been in use since decades (Tanskley, 1983). Being heritable entities, their association with economically important traits can be potential selection tools in crop breeding research. Broadly they may be classified into morphological, biochemical and molecular markers.Assessment of diversity has traditionally been through morphological characters, which has often found to be rather less effective.

2.3 Morphological Markers

Morphological markers are observable and that segregate with the trait(s) of interest. Sax (1923) first reported association of a simple inherited genetic marker with the quantitative trait in plants when he observed segregation for seed size associated with segregation of seed coat colour in beans. Plant breeders and geneticists extensively studied and employed the linkage between morphological markers and the economically important traits, which in most cases are quantitative in nature. Prior to the advent of isozymes and RFLP as markers, the major technique of gene location was the use of chromosome translocation. (Kang *et al.*, 1979) Once the location of major gene on a specific chromosome has been established, information on its linkages with flanking markers can be obtained via three-point cross linkage analysis.

Morphological traits or markers an however, very limited in number, environmentally influenced, stage specific expression, relative expressivity and penetrance, pleiotropy etc. Therefore, there was urgent need for more efficient marker system, possibly covering the whole genome without the limitations.

2.4 Biochemical Markers

Biochemical markers reveal polymorphism at protein level (Scandalios, 1969). In the 1980's, isozymes were first used as a biochemical marker system and used extensively to map some important traits. These markers have been used to identify QTL in maize, tomato, wild oats and soybeans (Stuber, 1992), variation in Korean jujube (Oh *et al.*, 1988). These markers gained greater success than the morphological markers. It eliminated the need for having gene markers with discrete and visible effects of the phenotype. However, isozyme analysis has its inherent disadvantages like limited number of enzyme loci, vulnerability to environmental influence and post-translation modifications and stage specific expression. Flavanoid pattern in *ber* has been studied to differentiate among genotypes by Vashishtha *et al.* (1989)

2.5 Molecular Markers

With the advent of molecular biological technique, DNA based markers have replaced enzyme markers in germplasm identification and characterization. DNA markers have distinct advantages over other markers as they are plastic, ubiquitous, stable and unlimited in number, discrete and non-deleterious, inherited in simple Mendelian fashion, covering the whole genome and have none of the problems experienced with morphological markers and free from epistatic and interaction and pleiotropic effects (Swathi *et al.*, 1999). The DNA markers are more widely employed for molecular tagging of genes and molecular marker assisted selection (Gupta *et al.*, 1999). In several crops, molecular markers closely linked to numerous traits of

economic importance have been developed (Caetano-Anolles and Gresshoff, 1997) that allows indirect selection for desirable traits in early segregating generations at the seedling stage by side stepping the confounding effect of the environment. Today various types of DNA markers are employed for identification and manipulation of genes governing agronomically important traits in many crop plants. Ideal DNA markers should have some desirable properties such as: Highly polymorphic nature, codominant inheritance (determination of homozygous and heterozygous states of diploid organisms), frequent occurrence in genome, selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices), easy access (availability), easy and fast assay, high reproducibility and easy exchange of data between laboratories.

The DNA markers systems have also been classified as the first second and third generation molecular marker systems. The first generation markers are mainly the RFLP and RAPD and their modifications, while the second generation molecular marker systems include AFLP, STMS and their modifications. Third generation molecular marker system are expressed sequence tags (Est`s) and the Single Nucleotide Polymorphisms (SNPs). DNA markers may be broadly classified into two types (i) *Hybridization based markers* and (ii) *PCR based markers*.

2.6 Hybridization Based Markers

These markers involve DNA: DNA hybridization, which in turn depends on base pairing rule. RFLP and VNTR fall under this category. Even though these markers are highly informative but require laborious procedures and use of hazardous radioisotopes.

2.6.1 Restriction fragment length polymorphism (RFLP)

It is one of the first marker systems developed and the technique is basically dependent on two steps *viz.*, restriction digestion and detection of specific fragment(s) using specific probe, Restriction endonuleases cleave total genomic DNA at specific sites, the digested DNA is then separated on Agarose gel and blotted to membrane and hybridized with radiolabeled specific probe, which detects specific homologous fragments. The membrane is then washed to remove the un-hybridized probes and subsequent detection of fragments by autoradiography (Botstein *et al.* 1980). RFLPs promise to be highly reproducible, codominant in nature, allowing differentiation of heterozygotes from homozygotes, multiple allelic forms and absence of pleiotropic effects on economic traits (Beckmann and Soller, 1983). Polymorphism detected by RFLP is basically due to the differences in position of restriction sites. Though its advantage on, it is time consuming technique, requires the use of radioactive isotopes and it has generally low level of polymorphism compared to the other PCR based marker systems. It also requires high quantities of good quality DNA and where only very limited amounts of source material are available, this requirement alone may

preclude its application. The use of single copy probes gives a simple banding pattern, which could be used easily for mapping and tagging genes of economic importance. A deviation from the basic RFLP is the use of multi-locus probes, which produce a complex pattern, and is ideal for DNA fingerprinting and individual identification. Two categories of such multi-locus probes are mainly used, *Minisatellites,* which is a tandem repeats of a basic motive of 10-60 bp (Jeffreys *et al.,* 1985) and *microsatellite*, which is repeats of a basic motive of 1-6 bp (Ali *et al.,* 1986).

Ragot and Hoisington (1993) compared the time efficiency and cost of RFLP (chemiluminiscent and radioactivity-based) and RAPD from simulation of maize genotyping experiments. The increase in total cost with increasing number of individuals genotyped and markers analyzed is higher for RAPD than for RFLPs. RAPDs generally were found to be more costly and time efficient for small sample size while RFLPs were suitable for bigger sample size. In RFLPs, cRFLP (chemiluminiscent) requires less exposure time than rRFLP (radioactivity-based) to obtain a given amount of information.

2.6.2 Variable number tandem repeat (VNTR)

The term VNTR refers to the variable sequence rather than to the method used to detect it (Nakamura *et al.*, 1987). These sequences are scattered at various locations in the human genome regions that are highly variable. Polymorphisms appear because of variation in the number of tandem repeats (VNTR loci) in a given repeat motif. Tandem repeats are multiple copies of a sequence of base pairs arranged in head to tail fashion. For example, a frequently found tandem repeat is CA, and one strand containing this type

of repeat reads CACACA, notated as (CA)n. The other strand would read GTGTGT... In this example, the number of repeating base pairs is two, but it can be more. When the repeating unit is less than four, the VNTR is called a microsatellite and when the repeating unit is longer it is a minisatellite. Short identical segments of DNA aligned head to tail in a repeating fashion are interspersed in the human genome. Some VNTR sequence segments are found at only a single locus in the human genome. Probes made of these sequences are single locus probes and yield patterns such as those at left when used to probe RFLP blots of DNAs of six individuals.

2.7 PCR Based Markers

The *in vitro* amplification of DNA by the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) has proven to be revolutionary technique in molecular biology. It facilitates *in-vitro* amplification of DNA by using two oligonucleotide primers complementary to opposing DNA strands. PCR is a rapid inexpensive and technically simple. PCR-based approaches are in demand because of their simplicity and requirement for only small quantities of sample DNA. By combining a thermostable DNA polymerase with repeated cycling through reaction conditions favoring DNA template denaturation, followed by template-primer annealing and finally primer extension, exponential DNA amplification occurs. Beginning with as little as one molecule of DNA, microgram quantities of a specific DNA fragment is produced in a couple of hours. Because of these features a multitude of new genotyping methods. The PCR based methods can be divided into two categories: (i) sequence-arbitrary methods, and (ii) those requiring a *priori* sequence information (Reiter, 2001).

2.7.1 Sequence-arbitrary primers

Because in many plant species (a) the need of availability of large amount of sequence information is limited in many species and obtaining additional sequence information can be cost-prohibitive, (b) based upon available sequence, the degree of polymorphism revealed by PCR amplification and fragment size separation is low, the development and use of sequence-arbitrary methods has occurred primarily with plant species. This include Random Amplified Polymorphic DNA (RAPD), Arbitrary Primed PCR (AP-PCR), DNA Amplification Fingerprinting (DAF), Inter Simple Sequence Repeat (ISSR) and Amplified Fragments Length Polymorphism (AFLP). These methods allow simultaneous assaying of 3-30 genomic sites. One drawback, however of these techniques is that the primers used are usually AT rich that amplify the heterochromatin region (non-genic region) more frequently. Moreover, annealing temperature is usually very low (37^0-40^0C) which provides for non-specific annealing to some extent and hence amplification may not be reproducible. AFLP and ISSR overcome this limitation by using highly stringent annealing conditions.

2.7.1.1 Random amplified polymorphic DNA (RAPD)

Amplification of genomic DNA, using single and short primer (10bp), under low stringency conditions (low annealing temperature due to the low primer Tm value *i.e.* 35-37^0C), results in multiple amplification products distributed randomly throughout the genome. This is called Random Amplified Polymorphic DNA (RAPD), (Williams *et al.,* 1990). This technique is widely accepted due to its simplicity and used extensively

for DNA fingerprinting, genome mapping (Williams *et al.,* 1990), gene tagging (Martin *et al.,* 1991, Paran *et al.,* 1991), population and polygenetic studies (Van Heusden and Bachmann, 1992) and varietal identification (Farooq *et al.*, 1995; Devanshi *et al.*, 2007) and genetic diversity analysis (Zhang *et al.*, 2005) in different food and fruit crops. There are a number of advantages of RAPD over the RFLP namely; simplicity, lower cost/sample, non-use of radioactivity and the higher level of polymorphism. On the other hand, reproducibility of RAPD has been a subject of considerable discussion as it generally has low reproducibility.

Principle of RAPD markers

The principle involved in generating RAPDs is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels and visualized by ethidium bromide staining. The use of a single 10-mer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and therefore cannot be used to identify

heterozygotes. Several factors have been shown to affect the number, size and intensity of bands. These include PCR buffers, dNTPs, Mg^{2+} concentration, cycling parameters, source of Taq polymerase, condition and concentration of DNA and primer concentration. Results obtained by RAPDs are highly prone to user error and bands obtained can vary considerably between different runs of the same sample. This limitation is, however, easy to overcome through practice and many articles have reported consistency in the profiles obtained from different runs of the same sample (Lerceteau *et al.*, 1997; Clerc *et al.*, 1998). The problem of the reproducibility of RAPDs data between laboratories that use different PCR machines may soon disappear with the availability of new improved PCR machines on the market. Mailer *et al.* (1994) have reported identical banding patterns obtained using different thermocyclers and success depends mainly on a defined set of conditions, which should be maintained constant when using different machines.

Impact of scoring error and reproducibility of RAPD data was estimated by Skroch and Nienhuis (1995) and they reported the probability of a scored RAPD band being scored in replicate data is strongly dependent on the uniformity of amplification conditions between experiments, as well as the relative amplification strength of the RAPD band. They observed significant improvement in the reproducibility of scored bands and some reduction in scoring error can be achieved by reducing differences in reaction conditions between replicates.

Other related techniques include Arbitrary Primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA Amplification Fingerprinting (DAF) (Caetano-Annoles *et*

al., 1991). These methods differ from RAPDs in primer length, the stringency conditions and the method of separation and detection of the fragments.

2.7.1.2 Arbitrary primed PCR (AP-PCR)

Arbitrary Primed PCR (AP-PCR) provides plus/minus DNA amplification based polymorphisms. In this technique, longer single primer is used (22-24bp) and primer concentrations are 10-fold higher (Welsh and McClelland, 1990). The thermal cycling profile begins with one or two cycles incorporating a low annealing temperature (40-48^{0}C typical) followed by 30 cycles using a high stringent annealing temperature and radiolabeled dCTP to label the newly synthesized fragments. In AP-PCR DNA fragments are size separated using PAGE and visualized via radiography (Welsh and McClelland 1990). It provides less number of bands detected as compared to RAPD and DAF.

2.7.1.3 DNA amplification fingerprinting (DAF)

In DNA Amplification Fingerprinting (Caetano-Anolles *et al.*, 1991), even shorter primers (5-7bp) at higher concentrations (3-30mM) with either low or high stringency annealing temperature are used, more number of fragments are detected. PAGE is used to resolve the amplified products with silver staining technique. It is an ideal system for individual identification and fingerprinting. Because of more DNA fragments, the likelihood of observing polymorphism between strains is increased.

2.7.1.4 Inter simple sequence repeats (ISSR)

This technique is not sequence arbitrary *per se*, but requires only limited sequence information and developed using micro-satellites themselves as primers. This involves the use of single primer composed of di-, tri-, tetra- or penta-nucleotide. It involves amplification of regions between adjacent, inversely oriented microsatellites using a single SSR containing primer (Zietkiwicz *et al.*, 1994). Polymorphisms are found abundant among species. These are dominant markers though occasionally a few of them exhibit codominance. Using this strategy, Gupta *et al.* (1994) found that tetra-nucleotide repeat primers provided informative and moderately complex patterns, tri-nucleotide less informative whereas, di-nucleotide primers resulted in smeared products. Only one primer is used in a PCR at a time. The primers can be either unanchored or anchored at 5' or 3' or at both the ends with 1-4 bases extended in the flanking sequences. When two such sequences are present within an amplifiable distance and in inverted orientation, the intervening DNA segment within these two repeats will be amplified. As the ISSR technique amplifies a large number of DNA fragments per reaction, representing multiple loci across the genome, it is an ideal method for fingerprinting rice varieties and a useful alternative to single locus or hybridization-based methods (Goodwin *et al.*, 1997). Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer *et al.*, 1993). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz *et al.*, 1994; Tsumara *et al.*, 1996; Nagaoka *et al.*, 1997). Each band corresponds to a DNA sequence delimited by two-inverted microsatellite. LikeRAPDs,

ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers.

The great robustness of ISSR in repeatability experiment and their being less prone to changing band patterns with changes in constituent or DNA template concentration makes them superior to other readily available marker systems in investigation of genetic variability variation among very closely related individuals and in crop cultivars classification (Fang and Roose, 1997; Nagaoka and Ogihara, 1997) and variability studies *e.g.* genetic diversity in *berchemia berchemiaefolia, a* Rhamnaceae plant (Lee *et al.* 2003) and *ber* (Singh *et al.*, 2007)

2.7.1.5 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP), is universally applicable and highly reproducible method, which reveals very high levels of polymorphism (Vos *et al.,* 1995). This technique is based on Selective Restriction Fragment Amplification of DNA fragments (SRFA), involving three steps (i) cutting genomic DNA with restriction enzyme(s), (ii) ligating double stranded adapters to restriction fragments, and (iii) amplifying selective restriction fragments using universal primers. During selective amplification, one of the paired primers is end labeled with ^{32}p. The amplified products are normally separated on a denaturing polyacrylamide gel and visualized by exposure to X- ray film, but the technique has been adapted to fluorescent labeled primers and also automated. SRFA can be performed with a single restriction enzyme, but the results are achieved when two different enzymes, a rare cutter (*e.g.* Mse I) and a

frequent cutter (*e.g.* Eco RI) are used. AFLP is a powerful, reliable, stable and rapid assay with potential application in genome mapping, DNA fingerprinting and marker assisted breeding. This PCR-based technique permits inspection of polymorphism at a large number of loci within a very short period of time and requires very small amounts of DNA. The reproducibility of AFLPs ensured by using restriction site-specific adapters and adapter-specific primers with variable number of selective nucleotides under stringent amplification conditions. Due to their various advantages, AFLPs are considered as one of the best molecular markers of choice for genome mapping. However, they require more DNA, dominant in nature, complex banding pattern, use of radioactivity and anchoring the randomly distributed amplified bands to particular chromosomal region and are quite difficult compared to STMS (described later). Probably the single greatest advantage of the AFLP technology is its sensitivity to polymorphism detection at the total-genome level. With all of these assets, AFLP markers are molecular standard for investigations ranging from systematics to population genetics. This technique has been used in genetic diversity studies in *Musa spp.* (Ude *et al.*, 2003), pear (Kim *et al.*, 2005), *Cucurbita pepo* (Ferriol *et al.*, 2003), Abyssinian banana (Negash *et al.*, 2002) *ber* (Singh *et al.*, 2006) and many other plant species.

2.8 Other Sequence Arbitrary Methods

Several variations of the above described methods are employed. Either by using more than one arbitrary primer (Callahan *et al.*, 1993) or by using a degenerate primer in the amplification reaction (Caetano-Anolles, 1994), increasingly complex RAPD and

DAF amplification patterns has been observed. One approach to find increased polymorphism is the pre-digestion of the template DNA with restriction endonucleases termed as template endonucleases cleavage multiple arbitrary amplicon profiling (tecMAAP). Despite the fact that endonucleases digestion destruct potential amplicons, new amplicons containing higher levels of primer-template mismatch are amplified. Another approach to enhance the level of informativeness from DAF reactions is by using primers which contain both a 5' mini-hairpin sequence and a short 3'arbitrary sequence either alone (Caetano-Anolles and Gresshoff, 1994) or in a two step amplification procedure called arbitrary signature from amplification profiles (ASAP) (Caetano-Anolles and Gresshoff, 1996). Using ASAP, a DAF amplification is first performed and then an aliquot of the resulting amplification reaction is used as template in a second amplification primed with either a mini-hairpin based primer or alternatively 5- anchored simple sequence repeat (SSR) primer (Caetano-Anolles and Gresshoff, 1996).

Two other methods have been described that combine aspects of RAPDs and SSRs. The first of these methods termed as random amplified microsatellite polymorphisms (RAMPs) (Wu *et al.*, 1994), is an extension of anchored SSR method described by Zietkiewicz *et al.*, (1994). By combining a radiolabeled, anchored SSR primer with a 10mer RAPD primer, informative fingerprinting patterns are observed. Because of the labeled anchored repeat primer, only those amplification products that contain the target repeat motifs are visualized. The other method involved hybridizing the SSR repeat primers to RAPD amplification patterns, is called either random

amplified hybridization microsatellites (RAHM) (Cifarelli *et al.*, 1995) or random amplified microsatellite polymorphisms (RAMPO) (Richardson *et al.*, 1995). In the technique, the bands observed following hybridization with the SSR probe were amplification products not observed following hybridization with the SSR probe were amplification product is not observed using only ethidium bromide staining (Richardson *et al.*, 1995) and thus may uncover additional polymorphism.

2.9 Sequence-Dependent Markers

These techniques are based on PCR using two sequence-dependent oligonucleotide primers. Sequence information is necessary in order to design primers, which would facilitate successful amplification of a specific DNA fragment. Despite their considerable development cost and sequence-dependent, these PCR-based markers are now being exploited in many plant species. Allelic and codominant nature, highly specific, reproducibility and assay robustness make these markers especially attractive for marker-assisted plant breeding. This includes Sequence Tagged Sites (STS), Sequence Characterized Amplified Region (SCAR), and Simple Sequence Repeats (SSR) otherwise referred as micro-satellites

2.9.1 Sequence tagged sites (STS)

A sequence tagged site (STS), is a short stretch of genomic sequence that can be detected by PCR and mapped to a specific site as a landmark in the genome. They are essentially derived from RFLP probe sequences by designing 20-25bp primers using these sequences (Oslon *et al.* 1989; Inoue *et al.*, 1994, Thomas and Scott 1993, Ribaut *et*

al.. 1997). They are co-dominant in nature and generally having lower level of polymorphism compared to microsatellites. A crucial event in the history of the Human Genome Project was the decision to use sequence-tagged sites (STSs) as common landmarks for genomic mapping. Following several years of constructing STS-based maps of ever-increasing detail, the emphasis has recently shifted towards large-scale genomic sequencing (Schuler, 1998). A computational procedure called 'electronic PCR' allows STS landmarks to be revealed as data emerge from the sequencing pipeline, thereby bridging the gap between mapping and sequencing activities. Electronic PCR (e-PCR) refers to the process of recovering these unique sites in DNA sequences by searching for subsequences that closely match the PCR primers and have the correct order, orientation, and spacing that they could plausibly prime the amplification of a PCR product of the correct molecular weight (Schuler, 1997). Hudson *et al.* (1995) constructed physical map of human genome using 15,086 sequence tagged sites (STSs) with an average spacing of 199 kilobases. In the project, assembly of a radiation hybrid map of the human genome containing 5,264 loci and incorporated 5264 loci. The map provides radiation hybrid coverage of 99 per cent and physical coverage of 94 per cent of the human genome.

2.9.2 Expressed sequence tags (Est`s)

Expressed sequence tags (Est`s) are derived mainly from cDNA sequences isolated from particular tissue or developmental stage or under biotic or abiotic stress. They are functional sequences; Primers are designed based on these sequences. This is co-dominant marker and it played a great role in construction of physical maps for

whole genome sequences. The increasing availability of Est`s in the rice genome project (Sasaki, 1998), wheat (Leigh *et al.*, 2003 and Nicot *et al.*, 2004) and grape (Scott *et al.*, 2000). They are abundant and distributed throughout the genome and could be a significant tool in gene mapping, tagging and cloning.

2.9.3 Sequence characterized amplified region (SCAR)

This is one modification of RAPD, which improves its reproducibility, SCAR (Sequence Characterized Amplified Region), in which the polymorphic band detected with RAPD are eluted, cloned and sequenced (Paran and Michelmore, 1993). Based on the sequence information, specific primer is then designed, which gives a highly reproducible banding pattern. The ability to observe polymorphism was dependent upon the SCAR. Some SCARs show dominant whereas, some other can show codominace (Paran and Michelmore, 1993). SCAR has succsfully been used for tagging of male fertility restorer gene in *Brassica juncea* (Ashutosh, 2007) and gene for resistence to *Plasmodium brassicae* in Chinese cabbage (Piao *et al.*, 2004)

2.9.4 Cleaved amplified polymorphic sequence (CAPS)

Instances, where direct gel electrophoresis of PCR products does not reveal polymorphism, may necessitate the use of more elaborate detection methods. CAPS are PCR amplification products which are subsequently cleaved using restriction endonucleases and size separated in order to reveal polymorphism (Konieczny and Ausubel, 1993). Due to the lower level of polymorphism detected using STS; cleaved amplified Polymorphic sequence (CAPS) is used as a STS modification for increasing

the level of polymorphism. The amplified product is digested with one or more suitable restriction enzyme(s) to detect polymorphism in the enzyme recognition site. CAPS has been done to unveil the polymorphism in *Brassica juncea* (Varshney *et al.*, 2004) and for tagging the gene for resistence to *Plasmodium brassicae* in Chinese cabbage (Piao *et al.*, 2004)

2.9.5 Single nucleotide polymorphism (SNP)

The single nucleotide polymorphism (SNP) refers to a specific and defined position at a chromosomal site at which the DNA sequences of two individuals differ by a single base. SNPs is the most abundant variation in the genome (Nasu *et al.*, 2002), they can contribute directly to a phenotype or can be associated with a phenotype as a result of linkage disequilibrium (Risch and Merikangas, 1996). SNPs are abundant, co-dominant in nature and highly reproducible systems. They may be found in both-transcribed and non-transcribed regions and in some instances are the direct cause for observed phenotypic variation. In humans, SNPs have been found at a frequency greater than 1 per 1000 base pairs. It has been used extensively to map, tag and cloning genes of interest (Monna *et al.* 2002). Nasu *et al.* 2002 has developed 213 SNP markers covering the entire rice genome using DNA sequences of three *japonica* cultivars, two *indica* cultivars and one wild rice *O.rufipogon.* The success of marker assisted selection (MAS) in the hands of plant breeders depends on the development of molecular marker systems that are not only more efficient and cost effective, but are also amenable to automation and high throughput approaches to handle large segregating populations. SNPs have proven ideal for this purpose (Gupta *et al.*, 2001).

SNPs may be found in the non-repetitive coding or regulatory sequences as well as the repetitive non-coding ones. It is important here to focus on SNP discovery in or nearby coding sequences, this will make it more likely that the SNPs will either be located within the gene of interest or will be tightly linked with it. Several approaches are employed for SNPs discovery including alignment of genomic and Est sequences available from more than one source. This is highly cost effective way. It is also very attractive for developing trait specific SNPs if the location and the sequence is available. A whole genome shotgun sequence is another approach for the discovery. In this approach, random clones from a mixture of DNA from several genotypes are sequenced for several folds after which the sequences are aligned. In case of organisms that have bees used for genome sequencing such as rice, one of the common approaches is the alignment of the overlapping BACs and PACs and detect the mismatched SNPs. Reduced representation shotgun (RRS) approach is yet another way for SNP discovery. In this approach, equal amounts of DNA from different genotypes are mixed, completely digested, electrophoressed and 500-600bp bands are excised, cloned and sequenced, alignment of the sequences from same loci will reveal the possible candidate SNPs for this particular loci. The second part of SNPs is the genotyping, which can be classified, in two broad approaches, gel-based and non gel-based SNP detection. The first part includes the use of RFLP or AFLP in case the SNP is altering restriction endonucleases recognition site, the use of Single Strand Confirmation Polymorphism (SSCP), allele specific amplification using allele specific primers is done. The non gel-base approach is the fast growing approach including *Taqman* assay, molecular

beacons, oligonucleotide ligation assay, DNA chips and microarrays, dynamic allele-specific hybridization, minisequencing and genetic bit analysis, temperature modulated heteroduplex analysis, *masscode*^{TM} system, *MegaBACE SnuPe* Genotyping Kit, Amersham Pharmacia Biotech, Inc, USA. And invasive cleavage assay

2.9.6 Simple sequence repeats (SSRs)

Microsatellite DNA sequences were first studied in humans, where they were found to be abundant and dispersed throughout the genome (Hamada and Kakunga 1982; Hamada *et al.* 1982). Since that time, they have been found in a wide array of other eukaryotes including several monocot and dicot plant species including rice. Microsatellites consist of tandemly arrayed di-, tri- and tetra-nucleotide repeats, and are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Among different crops species, the frequencies and occurrence of the most common dinucleotide repeats [$(AC)_n$ and $(GA)_n$] have been worked out in relatively greater detail. $(AAG)_n$ and $(AAT)_n$ are the most frequent trinucleotide repeats in plants (Gupta *et al.*, 1996). Human genome is estimated to contain on an average 10-fold more microsatellites than plant genome (Powell *et al.*, 1996). Microsatellites are not clustered in specific regions but rather uniformly distributed in different regions. Tomato is an exception in this regard as it shows clustering of microsatellite (Gupta and Varshney, 2000). Primers are designed using unique sequences on both sides of the flanking regions of the repeat motif region to avoid the complex multiple pattern, since these repeats are present up to thousand times throughout the genome, this ensures STMS to provide a simple banding pattern with a high level of polymorphism detected (Litt and

Lutty, 1989; Tautz, 1989; Weber and May, 1989) The high level of polymorphism detected is basically due to the difference of number of repeats in each individual. In several crop plants, including soybean (Akkaya *et al.*, 1992), rice (Wu and Tanksley, 1993; Akagi *et al.*, 1997), barley (Becker and Heun, 1995), wheat (Roder *et al.*, 1995), maize (Senior and Heum, 1993), brassica (Langercrantz, 1993) and fruit species like grapes (Scott *et al.*, 2000), citrus, specific amplification of microsatellite loci has indicated that microsatellite DNA markers are more variable than RFLP markers. This new type of molecular markers, known also as simple sequence repeats (SSR) or simple sequence length polymorphism (SSLP) has been developed based on DNA sequence variation, is based on a 1-6 nucleotide core element that is tandemly repeated from two to many thousands of times. (Hamada *et al.* 1982, Litt *et al.* 1989; Tautz *et al.* 1986; Stallings *et al.* 1991; Akkaya *et al.*, 1992) A different "allele" occurs at an SSR locus as a result of changes in the number of times a core element is repeated, altering the repeat region. They are co-dominant in nature, so very ideal for segregating populations. Differences in length at an S*TMS* locus are detected with DNA amplification by the PCR using two oligonucleotide primers that complement unique sequences flanking the SSR locus. Sizes of the amplified products are then precisely determined by electrophoresis in either agarose or Polyacrylamide gels with detection by EtBr staining, autoradiography (using a single ^{32}p- labeled primer) or fluorescence (using a fluorescent labeled primer). Current research has suggested that the length variations between alleles at an S*TMS* locus are created by slippage of DNA polymerase during the

replication of the tandem repeat followed by a failure of DNA mismatch repair enzymes to restore the original sequence (Strand *et al.,* 1993)

STMS can provide more information more easily than previous DNA-based genetic marker technologies, such as restriction fragment length polymorphism (RFLP) and randomly amplified DNA (RAPD). It is also sensitive, only a small quantity of DNA is required, analytically simple, data are unambiguously scored, and highly reproducible, broadly applicable, loci are frequently conserved between related species and sometimes across genera (Moore *et al.,* 1991), readily transferable, information can be communicated as simple sequences of primer pairs, and does not require the physical transfer of probes among laboratories ; flexible, these markers can be used as sequence tagged sites to provide anchors between genetic linkage maps and physical chromosome locations. The only significant limitation of SSR analysis may be the initial investment and the technical expertise required to clone and sequence the loci, which is not the case in rice since the whole genome sequence is now available. *In silico* identification of STMS loci becomes quite easy. Once primer sequences are designed and published, analysis of SSR loci will be practical for any laboratory capable of PCR and electrophoresis. The throughput and cost effectiveness of screening loci also could be greatly improved by multiplex PCR, which allows the simultaneous amplification and scoring of multiple STMS loci in a DNA sample in a single PCR reaction and a single lane of gel electrophoresis.

Through surveys of sequences in EMBL and Gene Bank databases, it was discovered that all possible di-and trinucleotide repeat motifs were present at 5- to 10-

fold greater frequencies than expected by a random distribution. The only exception being (CG)n which occurred at significantly less than a random frequency. Other forms of imperfect and scrambled arrangements of repeat units ("cryptic repeat") also were extremely common in eukaryotic genomes. Slippage during DNA replication was proposed as a mechanism for the creation and hypervariability of these repeat elements. Regions of divergence between conserved genes in closely related species often contained these simple repeats. It was hypothesized by Tautz (1989) that because STMS were so prevalent in the genome and so highly variable, mutations at these sites may be a more important source of evolutionary variation than classical point mutations and chromosomal translocations. The information content of STMS loci as a genetic marker is directly proportional to the number and frequency of alleles present in a population. It is known as the percentage of polymorphism or polymorphism information content (PIC) value. In a study of 100 $(AC)_n$ loci, Weber and May (1989), reported that 64% were perfect tandem repeats, 25% were imperfect, and 11% compound. The information content of the loci was directly correlated with the number of tandemly repeated units. PIC values ranged from close to 0 at n=10 up to 0.8 at n=24. As such, it was estimated that the human genome potentially contains ~7000 (AC)n loci with PIC >0.7.

2.10 Development of Microsatellite Markers

There are a number of ways to obtain microsatellite markers. Screening genomic libraries by hybridizing with SSR probes and sequencing the hybridized positive clones is traditional but a laborious and costly approach. While screening the SSR-enriched, small-

insert libraries can significantly reduce time and cost (Billotte *et al.*, 2001). Enrichment can be realized by hybridizing SSR-containing fragments with biotin-labeled probes that are either captured by magnetic beads coated with streptavidin or fixed on a nitrate filter (Edwards *et al.*, 1996). The eluted portion after removing non-hybridized DNA would be highly enriched for microsatellites, with enrichment efficiency between 50–90% (Butcher *et al.* 2000).

Ostrander *et al.* (1992) described an efficient method for the construction of small-insert genomic libraries enriched for highly polymorphic, simple sequence repeats. With this approach, libraries in which 40-50% of the members cotain $(CA)_n$ repeats are produced, representing an $\approx$ 50 fold enrichment over conventional small insert genomic libraries. Briefly a genomic library with an average insert size of less than 500 base pairs was constructed in a phagemid vector. Amplification of this library in a *dut ung* strain of *Escherichia coli* allowed the recovery of the library as closed circular single-stranded DNA with uracilfrequently incorporated in place of thymine. This DNA was used as template for second-strand DNA synthesis, primed with (CA)n or (TC)n oligonucletides, at elevated temperatures by a thermotable DNA polymerase. Transformation of this mixture into wild-type E. coli strains resulted in the recoveryof primer-extended products as a consequence of the strong genetselection against single-stranded uracil-contating DN molecules andrecovered a library that was enriched for targeted microsatellite-conating sequences. They suggested the use of this method to generate marker-selcted libarairies nearing any simple sequence repeat from cDNAa,

whole genomes, single chromosomes or more restricted chromosomal regions of interest.

Kandpal *et al.* (1994) described a simple and rapid method for constructing small-insert genomic libraries highly enriched for dimeric, trimeric and tetrameric nucleotide repeat motifs. This approach involves use of DNA inserts recovered by PCR amplification of a small-insert sonicated genomic phage library or a single-primer PCR amplification of *Mbo* I-digested and adaptor-ligated genomic DNA. The genomic DNA inserts are heat denatured and hybridized to a biotinylated oligonucelotide. The biotinylated hybrids are retained on a vectrex-avidin matrix and eluted specifically. The elute is PCR amplified and cloned. More than 90% of the clones in a library enriched for $(CA)_n$ microsatellites with this approach contained clones with inserts contating CA repeats. They also used this protocol for enrichment of $(CAG)_n$ and $(AGAT)_n$ sequence repeats and fot *Not* I jumping clones.

Li *et al.* (2001) constructed microsatellite-enriched libraries to isolate microsatellite sequences in *Avena* species and oat cultivars. One hundred clones were isolated and sequenced from three oat microsatellite-libraries enriched for either $(AC/TG)_n$, $(AG/TC)_n$ or $(AAG/TTC)_n$ repeats of these, seventy eight clones contained microsatellites. A database search showed that 42% of the microsatellite flanking sequences shared significant homology with various repetitive elements. *Alu* and retrotransposon sequences were the two largest groups associated with the microsatellites. Forty four primer sets were used to amplify the DNA from 12 *Avena* species and 20 *Avena sativa* cultivars. Sixty two percent of the primers revealed

polymorphism among the *Avena* species, but only 36% among the cultivars. In the cultivars, the microsatellites associated with repetitive elements were less polymorphic than those not associated with repetitive elements. Only 25% of the microsatellites associated with repetitive elements were polymorphic, while 46% of the microsatellites not associated with repetitive elements showed polymorphism in the cultivars.

Kolliker *et al.* (2001) developed simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). They sequenced 1123 clones from genomic libraries enriched for $(CA)_n$ repeat yielded 793 clones containing SSR loci. The majority of SSRs consisted of perfect dinucleotide repeats, only 7% being trinucleotide repeats. After exclusion of redundant sequences and SSR loci with less than 25 bp of flanking sequence, 397 potentially useful SSRs remained.

Aranzana *et al.* (2002) developed a genomic libaray enriched with AG/CT repeats from the peach cultivar 'Merrill O'Henry' and observed that 61% of the clones carrying a microsatellite sequences and a yield of one polymorphic microsatellite every 2.17 sequenced clones. From 35 microsatellite detected, 24 were polymorphic in a set of 25 cultivars. A total of 82 alleles were found with the polymorphic microsatellites with an average of 37% of observed heterozygosity.

He *et al.* (2003a) developed microsatellite markers in cultivated peanut using the SSR enrichment procedure and observed that the GA/CT repeat was the most frequently dispersed microsatellite in peanut. The primer pairs were designed for fifty-six different microsatellites, 19 of which showed a polymorphism among the genotypes studied.

Ahmad *et al.* (2003) evaluated the potential of microsatellite markers for use in *Citrus* genome analysis. Microsatellite loci were identified by screening enriched and nonenriched libraries developed from 'Washington Navel' *Citrus.* Microsatellite-containing clones were sequenced and 26 specific PCR primers were selected for cross-species amplification and identification of cultivars/clones in *Citrus.* After an enrichment procedure, on average 69.9% of clones contained dinucleotide repeats (CA)n and (CT)n, in contrast to <25% of the clones that were identified as positive in hybridization screening of a nonenriched library. A library enriched for trinucleotide (CTT)n contained <15% of the clones with (CTT)n repeats. Repeat length for most of the dinucleotide microsatellites was in the range of 10 to 30 units. We observed that enrichment procedure pulled out more of the (CA)n repeats than (CT)n repeats from the *Citrus* genome. All microsatellites were polymorphic except one. No correlation was observed between the number of alleles and the number of microsatellite repeats.

Ritschel *et al.* (2004) developed microsatellite markers using enriched genomic library in melon (*Cucumis melo* L.) and cucurbit species. They identified seven hundred clones containing microsatellite sequences from a Tsp-AG/TC microsatellite enriched library, from which they designed one-hundred and forty-four primer pairs. When 67 microsatellite markers were tested on a panel of melon and other cucurbit accessions, 65 revealed DNA polymorphisms among the melon accessions. For some cucurbit species, such as Cucumis sativus, up to 50% of the melon microsatellite markers could be readily used for DNA polymophism assessment, representing a significant reduction of marker development costs.

Giraldo *et al.* (2005) developed 26 polymorphic microstallites from a genomic library of fig (*Ficus carica* L., Moraceae, cv. Cuello de Dama Blanco) enriched for CT/AG repeats and found that enrichment procedure was highly successful (>60% of the clones sequenced contained microsatellite sequences). Microsatellite polymorphism was evaluated in 15 fig cultivars from different geographical areas. The mean expected and observed heterozygosities over the 25 single-locus microsatellites averaged 0.42 (range of 0.12-0.77) and 0.47 (range of 0.13-0.93) respectively. The total value for the probabaility of identity was 2.81×10^{-8}. The low values of variability and the absence of clear groups in the genotypes studied and indicated a narrow genetic base in cultivated common fig. The developed microsatellite primers were also observed for high level of cross-species transferabaility in *Morus species*.

2.11 Use of Microsatellite Markers

The application of STMS marker analysis to plant genetics is only just beginning, but is being adopted very rapidly throughout the research community. A number of loci have been characterized in agricultural species including rice, soybean, maize, barley, rapeseed, and grape. In each case, preliminary work involved identifying STMS loci in existing sequence database, creating primer pairs for these loci, and surveying a small set of diverse germplasm for polymorphism. Subsequent efforts have involved cloning and sequencing STMS loci by screening size-fractionated genomic libraries, and using primer pairs flanking STMS loci to survey sets of individuals representing both agronomically important and distantly related species.

The analysis of STMS alleles in plant DNA is being greatly advanced by the capabilities for automated sizing of PCR products by GeneScan™ fluorescence-based detection (Applied Biosystems Division of Perkin-Elmer). Differences in allele sizes of only two nucleotides can be resolved reproducibly. A multiplex of different primer pairs tagged with fluorescent dyes of different colors allows multiple loci to be analyzed in a single PCR reaction and a single gel lane. This technology ultimately may be complemented with automated DNA extraction and PCR set-up, permitting very high rates of sample throughput and low unit cost for large scale operations, such as genetic resources profiling or scoring segregation in marker assisted breeding programs. STMSs will be useful in the study of genetic organization and variation in a myriad of ways. Their ease of use and high information content naturally will lead to the complementation and replacement of other types of genetic markers in many situations, but possibly also to novel applications not previously considered for DNA-based markers (Brown *et al.*, 1996). The application of STMS markers to varietal identification and for plant breeders and seed producers' right protection is already underway (Smith, 1994).

Identification of markers linked to useful traits has been based on construction of complete linkage maps and the study of co-segregation, or bulk segregation analysis (BSA) in case of simple traits. However, alternative methods such as the construction of partial maps and combination of pedigree and marker information have also proved useful in identifying marker/trait association. Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become

extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it. Microsatellite markers, especially STMS markers, have been found to be extremely useful in this regard. Owing to their quality of following clear Mendelian inheritance, they can be easily used in the construction of linkage maps, which can provide an anchor or reference point for specific regions of the genome.

Allele mining focuses on the detection of allelic variation in important genes and/or traits within a germplasm collection (Simko *et al.*, 2004). If the targeted DNA (either a gene of known function or a given sequence) is known, then the allelic variation (usually point mutations) in a collection can be identified, using methods developed for the purpose. (Lemieux *et al.*, 1998). Association studies of artificial progenies are an alternative to segregation analysis for identifying useful genes by correlation of molecular markers and a specific phenotype (Gebhardt *et al.*, 2004). Association studies can be performed on a germplasm collection and also on other materials, as long as significant linkage disequilibrium (LD) exists, for example, breeding materials. It may be especially useful for those crops where appropriate populations for genetic analysis cannot be obtained or their production is too time-consuming (Simko *et al.*, 2004). It is also useful for those crops for which sequence information does not exist and is unlikely

to be available soon. Knowledge of molecular information in major crops and species and of the synteny of genomes, especially conservation of gene order, has also opened up prospects for identifying important genes or variants in other crop types, particularly those that receive little attention from formal research.

2.12 Genetic Diversity Studies in *Ber*

Genetic variability is a pre-requisite for genetic improvement in any breeding programme. Since reports on genetic diversity studies on *Ziziphus* using molecular markes are very scanty (Devanshi *et al.*, 2007), in the review the fruit and woody trees in general will be detailed for the molecular studies. Molecular marker technology has wide and diverse applications they can broadly be classified into two main categories. The first category is genome analysis applications, which include mapping, tagging, map based cloning, gene pyramiding and marker-assisted selection (MAS) for genes of interest in both simple as well as quantitative traits (Wu and Tanksley, 1993). The second category is the fingerprinting applications including varietal identification, ensuring seed purity, phylogeny and evolution studies, diversity analysis, elimination of germplasm duplicates .The DNA markers are more widely employed for molecular tagging of genes and molecular marker assisted selection (Gupta *et al.*, 1999). Microsatellite markers have been used to distinguish the inter-varietal chromosome substitution lines of wheat (Korzun *et al.*, 1997).

Prevost and Wilkinson (1999) tested two statstical functions, marker index and resolving power for their correlation to the proportion of genotypes identified but is

independent of the number of the genotypes studied in their study on fingerprinting of potato cultivars using ISSR markers. Marker index failed to correlate significantly with genotype diagnosis but a strong and seemingly linear relationship was observed between resolving power of a primer and its ability to distinguish genotypes (r^2=0.98). Resolving power of one or a pair of primer was found to provide amoderately accurate estimate of the number of genotypes identified. The marker index did not show any significant relationship between MI and the number of genotypes identified whereas, a linear relationhsip was observed between Rp and the proportion of cultivars identified.

Oh *et al.* (1988) suggested that Korean jujube (*Z. sativa*) strains could be classified with allozyme analysis using glutamate oxalacetate transaminase (GOT), esterase and peroxidase systems. Further analysis, however, showed that use of allozymes did not always show satisfactory results.

Vashishtha *et al.* (1989b) analysed the flavonoid distribution patterns of 59 *ber* cultivars along with the wild relatives, jhar (*Z. nummularia*) and boradi (*Z. mauritiana* var. *rotundifolia*). While no flavonoids in the wild relatives were observed to be common with those in the *Z. mauritiana* cultivars, similarities were recognised among the *ber* cultivars. The flavonoid patterns of many of the cultivars did not agree with the observed relationships based on their morphological characteristics, *e.g.* cultivars Ajmeri, Umran and Katha had identical flavonoid patterns and morphological characters, whereas round fruited cultivars such as Gola, Gola Gurgaon, Popular Gola and Kakrola Gola were dissimilar in their flavonoid patterns. Based on the distinctly different flavonoid patterns,

cultivars Illaichi, Willaiti, Mirchia and Sanaur-3 were recognised as chemical races (Vashishtha *et al.*, 1989a).

Weekly *et al.* (2002) studied genetic diversity in *Z. celeta using* random amplified polymorphic DNAs (RAPDs) to investigate genetic variability. One hundred and ninety-nine unique stem samples collected from one *ex situ* and five *in situ* populations were assayed for the presence or absence of a band for 32 RAPD markers. Based on unweighted pair-group mean cluster analysis (UPGMA), only 11 multi-locus genotypes (MLGs) were identified. Eight of these MLGs correspond to MLGs identified in an earlier allozyme study.

Meghala *et al.* (2005) studied genetic diversity among Indian sapota (*Manilkara zapota*) cultivars using RAPD markers and reported high genetic variability in Indian sapota cultivars might have originated through seedling segregation, intercrossing among cultivars or because a large number of cultivars/genotypes were introduced. It is also possible that both of these factors might have contributed to the genetic diversity in this crop.

Koller *et al.* (1993) differentiated 11 apple cultivars using random amplified polymorphic DNA (RAPD) markers and suggested that it would be possible to establish a standard set of primers that can be used to distinguish and characterize most of the common apple cultivars.

Schnell (1993) analysed 9 *Mangifera species* to determine phylogenetic relationship using 10 RAPD markers. One hundred nine bands were resolved and the

cluster analysis suggested that within *Mangifera* section, *M. casturi*, *M. quardifida* and *M. torquenda* are closely related, but *M. indica* and *M. laurina* are quite distant from the others. Within the subgenus Limus, *M. foetida* and *M. pajang* are genetically close; *M. decandra* and *M. odorata* are more distant.

Stiles *et al.* (1993) analysed relationships among ten cultivars of papaya (*Carica papaya* L.) using RAPD markers. The 11 RAPD primers amplified a total of 102 distinct fragments. The genetic similarity coefficient ranged from 0.7 to 0.95 that suggested a narrow genetic base for domesticated papaya.

Jarret *et al.* (1997) analysed simple sequence repeat markers in 33 watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) accessions to study genetic relationship. They reported substantial increase in the ability to differentiate among these accessions by using SSR markers as compared to enzyme polymorphism (Biles *et al.*, 1989) but similar to RAPD polymorphism (Lee *et al.*, 1996).

Fang *et al.* (1997) worked on fingerprinting of trifoliate orange germplasm accessions with isozyme, RFLP and inter-simple sequence repeat markers and reported that ISSR markers offer great potential for differentiating closely related citrus cultivars. They suggested that it may be possible to differentiate most mutationally derived citrus cultivars by using ISSR markers as probes in RFLP analysis.

Fang *et al.* (1998) used ISSR markers to study phylogenetic relationships among 46 *Citrus* L. accessions representing 35 species. The 10 primers generated 642 polymorphic fragments and grouped the 46 accessions into five clusters. Relationships

among the studied Citrus accessions revealed by the ISSR markers were in agreement to the previous taxonomic classifications.

Huang and Sun (2000) studied genetic diversity and relationships of 40 accessions of *Ipomoea*, representing 10 species of series of *Batatas* using ISSR markers. On average, 52 bands per accession were generated with most of the primers containing dinucleotide repeats. The ISSR analysis showed 62.2% polymorphism among the 40 accession studied. Of the species, examined *I. trifida* was found to be the most closely related to cultivated sweetpotato, the hexaploid *I. batatas*, while *I. ramosissima* and *I. umbraticola* were the most distantly related to *I.batatas* within the series.

Luro *et al.* (2000) studied genetic diversity among *Citrus* species using simple sequence repeats (SSRs). In the study, the genetic relationships among 73 individuals of 8 more economically important species of the *Citrus* genus (*C. reticulata Blanco, C. sinensis* (L.) Osb., *C. aurantium* L., *C. paradisi* Macf., *C. maxima* (Burm.) Merr., *C. limon* (L.) Burm., *C. aurantifolia* (Christm.) Swing. and *C. medica* L.) were estimated by the amplification of 10 loci of SSR. Polymorphism of 8 STMS was sufficient so as to obtain genetic aggregations where three main groups were clearly identified: the orange-mandarin group, the pummelo-grapefruit group and the lime-citron-lemon group. These *Citrus* species relationships were in large agreement with those established by previous analyses based on molecular markers and morphological traits, suggesting that SSR data will be useful for exploring infra-generic *Citrus* genetic taxonomy.

Degani *et al.* (2001) studied genetic relationship in strawberry using AFLP and RAPD markers and observed that RAPD markers successfully distinguished between 41 strawberry cultivars. They reported that RAPD-derived genetic similarity values to be better correlated with the coefficients of ancestory than the AFLP-derived genetic similarity values and suggested selective use of certain AFLP primer pairs which could be shown to give good genome coverage in strawberry might improve the utility of AFLP markers for predicting genetic relationships.

Kolliker *et al.* (2001) designed and characterize primers for 117 SSR loci and PCR products in the expected size range were amplified from 101 loci. These markers are highly polymorphic. 88% detecting polymorphism across seven white clover genotypes with an average allele number 4.8. Four primer pairs were tested in F_2 population revealing Mendelian segregation. Successful cross-species amplification was achieved in at least one out of eight legume species for 46 of 54 primer pairs, although rate of successful amplification was significantly higher for *Trifolium* species when compared to species of other genera.

Li *et al.* (2001) evaluated level of polymorphism of microsatellite developed by them in *Avena* species and oat cultivars. An average of four alleles with polymorphism information content (PIC) of 0.57 per primer set was detected among the Avena species, and 3.8 alleles with a PIC of 0.55 among the cultivars. In addition, 54 barley microsatellite primers were tested in *Avena* species and 26% of the primers amplified microsatellites from oat.

Herrera *et al.* (2002) characterize genetic variation between *Vitis vinifera* cultivars using RAPD and ISSR markers. Both the marker system distinusihed between the cultivar studied, although the resolving power of ISSR profiles was higher than that of RAPDs. They suggested use ISSR as an alternative cheap and technically simple approach that has high levels of repeatability.

Ahmad *et al.* (2003) observed 118 putative alleles generated through newly developed 26 SSR primer pairs using microsatellite enrichment library method in *Citrus*. The number of putative alleles per primer pair ranged from 1 to 9 with an average of 4.5. Microsatellite markers discriminated sweet oranges [*Citrus sinensis* (L.) osb], mandarin (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* Macf.), lemon [*Citrus limon* (L.) Burm.f.], and citrange (hybrids of trifoliate orange and sweet orange), at the species level, but individual cultivars/clones within sweet oranges, mandarins and grapefruit known to have evolved by somatic mutation remained undistinguishable. They suggested that since these microsatellite markers were conserved within different *Citrus* species, they could be used for linkage mapping, evolutionary and taxonomic study in *Citrus*.

He *et al.* (2003a) in their study on peanut microsatellite primers observed the average number of amplified alleles per locus was 4.25, and up to 14 alleles were found at one locus. He *et al* (2003a) confirmed that microsatellite DNA markers produce a higher level of DNA polymorphism than other DNA markers in cultivated peanut.

He *et al.* (2003b) generated five hundred DNA sequences of tomato and searched for SSRs in these to design PCR primers. They screened 158 pairs of SSR primers screened against a set of 19 diverse tomato cultivars, 129 pairs produced the expected DNA fragments in their PCR products, and 65 of them were polymorphic with the polymorphism information content (PIC) ranging from 0.09 to 0.67. Among the polymorphic loci, 2-6 SSR alleles were detected for each locus with an average of 2.7 alleles per locus; 49.2% of these loci had two alleles and 33.8% had three alleles. The vast majority (93.8%) of the microsatellite loci contained di- or tri-nucleotide repeats and only 6.2% had tetra- and penta-nucleotide repeats. It was also found that TA/AT was the most frequent type of repeat, and the polymorphism information content (PIC) was positively correlated with the number of repeats. The set of 19 tomato cultivars were clustered based on the banding patterns generated by the 65 polymorphic SSR loci. Since the markers developed in this study are primarily from expressed sequences, they can be used not only for molecular mapping, cultivar identification and marker-assisted selection, but for identifying gene-trait relations in tomato.

Mengjun (2003) studied genetic diversity in chinese jujube (*Ziziphus jujuba* Mill.), and observed that it has high diversity in the levels such as chromosome karyotype (1A, 2A, 3A, 1B, 2B, 2C); shape, size (11.5-22.1μ×21.0-26.3μ) and surface sculpture of pollen; leaf length (2.3-10.1cm) and flower diameter (2.9-10mm); especially the shape, colour, weight (2-46g), growth period (60-145d), soluble solid (17-45%), Vc (61-1174mg/100g.fw) and cAMP content (2-302nmol/g.fw) of fruit; as well as the RAPD and

isoenzyme (7 kinds) patterns. Diversity within some cultivars was also revealed by RAPD.

Ude *et al.* (2003) used 15 AFLP primer pairs and 60 RAPD primers to detect polymorphism and access genetic relationships in 25 plantains (*Musa spp.* subgroup AAB) from diverse parts of Western and Central Africa. They reported superiority of AFLP technique over RAPD technique based on greater PIC values for the primers of the former. A small group of cultivars of Cameroon were separated from the bulk of the plantains and based on that suggested to harbour useful genes from Cameroon for widening the genetic base of breeding population derived from the plantains.

Awasthi *et al.* (2004) studied genetic diversity and interrelationships among twelve domesticated and three wild species of the genus *Morus* (mulberry) using RAPD and ISSR markers. They observed that RAPD analysis using 19 random primers generated 128 discrete markers ranging from 500-3000 bp in size. One-hundred-nineteen of these were polymorphic (92%), with an average of 6.26 markers per primer. Among these were a few putative species-specific amplification products which could be useful for germplasm classification and introgression studies. The ISSR analysis employed six anchored primers, 4 of which generated 93 polymorphic markers with an average of 23.25 markers per primer. Cluster analysis of RAPD and ISSR data using the WINBOOT package to calculate the Dice coefficient resulted into two clusters, one comprising polyploid wild species and the other with domesticated (mostly diploid) species.

Ritschel *et al.* (2004) tested a random sample of 25 microsatellite markers to characterize 40 accessions of melon, generating an allelic frequency database for the species. The average expected heterozygosity was 0.52, varying from 0.45 to 0.70, indicating that a small set of selected markers should be sufficient to solve questions regarding genotype identity and variety protection. Genetic distances based on microsatellite polymorphism were congruent with data obtained from RAPD marker analysis.

Zhuang *et al.* (2004) studied genetic relatedness among Cucumis spp. using SSR and RAPD marker systems. They observed a total of 109 SSR and 398R RAPD bands and analysed these for genetic relationships thorugh cluster analysis. From the study they reported that these two marker systems are highly concordant with correlation between SSR and RAPD genetic distance (GD) estimates r = 0.94. SSR and RAPD analysis of 22 accessions grouped them into two distinct grouped and designated these as CS and CM. Within the CS group, 11 *C. sativus* genotypes, *C. hytivus* and *C. hystrix* accessions, whereas, in the group CM six *C. melo* genotypes and *C. metuliferus* were grouped.

Zhang *et al.* (2005) tested RAPD markers for relationship studies in tobacco (*Nicotiana tabacum* L.) cultivars and observed clear pattern of divison among the flue-coloured tobacco accessions based on geographic origin. They reported that RAPD is an effective tool for flue-coloured tobacco germplasm management, cultivar protection and cultivar improvement.

Singh *et al.*, (2006b) studied genetic diversity among *ber* (*Ziziphus* spp.) genotypes using AFLP markers. They reported that 11 primer pairs detected 952

fragments of which 789 (83.8%) were polymorphic. Similarity coefficients ranging from 0.14-0.86 suggested the divergence among *Ziziphus* accessions. Cluster analysis revealed complete separation of the accessions of the cultivated and the wild species into two distinct groups. Morphologically similar cultivars often remain clustered together with high degree of similarity. High correlation of the similarity matrix obtained with a single primer combination with that based on all the eleven primer combinations suggested utility of one or a few primer combinations in the estimation of diversity in *ber*. Nine of the primer combinations detected many accession specific amplified fragments and individually gave a discrimination rate of 1. Any one of these informative primers thus can be used for maintenance of the quality of both the scions and the rootstocks for establishing productive orchards.

Devanshi *et al.* (2007) studied genetic relationship among 50 *ber* genotypes representing *Z. mauritiana*, *Z. nummularia* and *Z. spina-christi* using RAPD markers. Out of 120 primers initially tested, 46 were highly reproducible and generated 368 RAPD markers with 86.2% polymorphism (316 polymorphic bands). The number of amplification product per primer ranged from 2 to 17 with an average of 8 bands per primer. The resolving power (Rp) for different primers ranged from 0.48 to 9.37 and polymorphic information content (PIC) ranged from 0.12 to 0.82. Nineteen primers distinguished at least one genotype that would prove to be highly useful for identification of genotype and designing future breeding strategy. Genetic relationships between the accessions were established based on Jacquard's similarity coefficient and it ranged from 26.3% to 78.9% suggesting that the *Ziziphus* germplasm is genetically diverse. The

present study has proved that *ber* genotype earlier reported to be similar based on morphology are genetically different. They suggested that RAPD analysis is an efficient marker technology for delineating genetic relationships among genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategy for conservation and improvement programmes.

Singh *et al.* (2007a) studied bitter gourd (*Momordica charantia* L.) for the genetic diversity based on inter-simple sequence repeat (ISSR) markers and reported the number of polymorphic markers ranged from 0 (UBC 841) to 12 (UBC 890) with a mean of 6.27 markers per primer. Pair-wise genetic distances (GD) of the 38 bitter gourd accessions, based on the 125 markers, ranged from 0.093 ('Pusa Do Mausami'- green *vs.* 'DBTG 7') to 0.516 ('Pusa Do Mausami'- white *vs.*'DBTG 101') and suggested a wide genetic base for these genotypes.

Singh *et al.* (2007b) studied genetic diversity among 47 *ber* accessions belonging to cultivated species (*Ziziphus mauritiana* Lam) and one wild accession of *Ziziphus nummularia (Burm f)* using inter-simple sequence repeat (ISSR) markers. A total of 167 amplification products were detected with 18 ISSR primers of which 152 (89.96%) were polymorphic. Most of the primers that produced distinct bands (14 primers out of 18) contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more polymorphic bands. Genetic similarity ranging from 43.07% to 90.30% suggested that the 48 *Ziziphus* genotypes used in the study were divergent. Cluster analysis based on UPGMA method and Bootstrap analysis separated all the 48 genotypes in four distinct clusters. They reported that morphologically similar genotypes can be distinguished and

emphasized the use of molecular markers to the taxonomists. Morphologically similar but genetically distinct genotypes, identified using ISSR markers could be potential sources for genotype identification and to resolve controversies over misnomination of *ber* genotypes.

Chapter 3

MATERIALS AND METHODS

The present study on "Characterization of *Ziziphus* by DNA Fingerprinting" was undertaken on a collection of forty eight genotypes of *ber* including *Ziziphus mauritiana* L. and *Ziziphus nummularia* (Table 2) using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers. Genotypes were comprised of cultivars (exotic and indigenous) and inter-varietals hybrids developed at Indian Agricultural Research Institute, New Delhi (India). In addition, the study aimed to develop microsatellite markers from partial genomic library and the validation of these markers on the 48 *ber* genotypes studied for characterization through RAPD and ISSR markers. The materials and methods of the study are described under following heads:

3.1 Location of the Experimental Sites

The experiment was carried out under National Agricultural Technology Project at of Division of Fruits and Horticultural Technology, Indian Agriculture Research Institute, New Delhi located in 28°38′14.62′′N and 77°12′39.20′′ with an elevation of 713 ft from the sea level. Molecular study on DNA characterization of *Ziziphus* work was done at Functional Genomics Laboratory, National Research Centre on Plant Biotechnology (NRCPB), Indian Agricultural Research Institute, New Delhi. Expenditure incurred to carry out this work was met from this NATP project.

3.2 Materials and Equipments

Plant material and other facilities utilized are written under different subheads:

3.2.1 Plant material

In *ber*, morphological differences have been observed for tree features, flavour and fruit shape (Fig 1). Leaves of 48 genotypes representing *Ziziphus mauritiana and*

Ziziphus nummularia were obtained from germplasm collections maintained at Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi and Central Institute for Tropical Horticulture, Bikaner, India. Pruned trees sprouted in the month of June and then young and healthy leaves were harvested and immediately stored at -80° C until total genomic DNA was extracted.

Fig 1: Variation in fruit shape in *ber* genotype

3.2.2 Facilities for molecular characterization

For raising the genotypes: Fruit orchard and field facilities

Equipments: Electronic balance, Centrifuge, Microfuge, Water bath, Refrigerator, BOD incubator, Deep Freezer (-20° C and –80° C), Mortar and pestle, Heater-magnetic vortex, magnetic stirrer, Incubator, Heater, Oven, Spectrophotometer, Fume-hood, glassware (beakers and flasks of different volumes), PCR machine, Horizontal gel electrophoresis

assembly, Vertical gel electrophoresis assembly, Gel documentation assembly and micropipettes (0.5-10 µl, 2-20 µl, 20-200 µl, 100-1000 µl,).

Chemicals used for molecular characterization: Chemicals used with the sources are listed in Appendix I and Appendix II.

3.3 Methods for Analysis

3.3.1 Molecular analysis

3.3.1.1 Isolation, purification and quantification of genomic DNA

DNA isolation: Extraction of genomic DNA was carried out by CTAB (Cetyl-tetramethyl ammonium bromide) method as described by Doyle and Doyle (1990) with minor modifications. For each variety, 2 to 3 g of young healthy leaves were taken and cleaned with moist tissue paper. The leaf material was placed in a pre-cooled mortar, frozen by adding liquid nitrogen and crushed vigorously with a pestle to fine powder. The care was taken to prevent thawing of the material. The powder was immediately transferred to an autoclaved 50 ml centrifuge tube containing 15 ml of pre-heated (at 65°C for 30 min) DNA extraction buffer. The mixture was vortexed well and incubated at 65° C in water bath for 45 to 60 min with intermittent swirling. After incubation the tubes are kept at room temperature for 10 min and then an equal volume of chloroform : isoamyl alcohol (24:1) was added and mixed by gentle swirling for 15 to 20 min. Mixture was then spun at 15,000 rpm for 20 minutes at room temperature. The upper aqueous phase was transferred to a fresh centrifuge tube with a wide born tip to avoid DNA shearing and equal volume of chilled isopropanol was added. The contents

were mixed by gentle inversions. The tubes were left for overnight at 4°C. The precipitated DNA so obtained was pooled out using wide born tips, which were then spun at 10,000 rpm for 10 minutes. The aqueous part was decanted. The pellet was washed with 70% ethanol. Then, the tubes were spun at 10,000 rpm for 10 min at 25° C. The aqueous part was decanted and dried under vacuum or at 37° C for one hour. DNA pellet was dissolved in 500 µl TE (10:1) buffer (pH 8.0). Presence and quality (whether there is shearing or not) of crude DNA in the extracted samples was tested using 0.8% agarose gels before pushing ahead with the purification.

DNA purification: All samples of crude DNA were treated with RNaseA (10 mg/ml) at a concentration of 5 µl/ g of leaf tissue used for extraction and mixed by gentle inversion and then incubated for an hour at 37° C. Equal volume of PCI (phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1) solution was added to the mixture, gently mixed by swirling for five minutes. Mixture was spun at 10,000 rpm for 10 minutes and the aqueous phase was taken out in a new 1.5 ml eppendorf tube. Equal volume of Chloroform and iso-amyl alcohol 24:1 was added to the aqueous phase, mixed gently for five minutes and spun at 10,000 rpm. Chloroform: isoamyl alcohol (24:1) extraction step is repeated. The purified DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.6), keeping for 5 min at room temperature and then by adding 2.5 times (v/v) chilled ethanol (95%). The mixture was mixed gently and incubated at –20° C for 2 hr, centrifuged for 5 min at 10,000 rpm, supernatant discarded and pellet washed twice with chilled 70% ethanol. Extra salts were removed by further washing with 70% ethanol. The DNA was pelleted and dried under vacuum. The pellet was dissolved

in a minimum volume of 100 µl TE (10:1) buffer pH 8.0 at room temperature and stored at -20° C.

DNA quantification: DNA quantification was done by two methods for authentification of quantity per sample. The first method was performed using a Hoefer Dynaquant 200 flourimeter. It works on the principle of measuring fluorescence emitted by the double standard DNA-Hoechst 33258 dye complex, which is directly proportionate to the amount of DNA in the sample. The flourimeter was calibrated with 2 µl calf thymus DNA solution (100 µg/ml) as standard added to 2 ml of assay solution. Then, the concentration of unknown sample was measured directly in ng/ml by adding 2 µl of genomic DNA sample to 2 ml of assay solution.

The second method used was gel-assay method. The samples were loaded on 0.8% agarose gel in 0.5X TAE buffer and using diluted λ uncut genomic DNA as standard. The intensity of individual samples was compared with 50 ηg λ uncut. As per the band intensity, samples were diluted with appropriate amount of sterilized distilled water to yield a working concentration of 10 ηg/µl and again loaded on 0.8% gels until all the samples finally reach the uniformity and stored at 4°C. PCR was performed to test the accuracy of quantification

3.3.1.2 Optimization of conditions for RAPD

The procedure described by Williams *et al.* (1990) was used for carrying out PCR reaction with minor modifications to produce RAPD profiles. The PCR mixture consisted of *Taq* DNA polymerase, PCR buffer, dNTPs, $MgCl_2$, oligonucleotide primers

(10 nucleotides long) and genomic DNA. Optimization of PCR component concentration was carried out for *Taq* DNA polymerase, $MgCl_2$ and primer as given below in Table 3 The concentrations of genomic DNA, dNTPs and PCR buffer did not vary. PCR was carried out in Perkin Elmer 9,600 Thermocycler (USA).

Table 2: Component concentrations used for optimization of PCR mixture

Treatment	*Taq* DNA polymerase (U)	$MgCl_2$ (mM)	Primer (μM)
1	0.5	2	1
2	0.5	1	2
3	0.75	2	1
4	0.75	1	2
5	1.00	2	1
6	1.00	1	2

Thermocycling conditions were as follows:

1. Denaturation at 94° C for 5 min,
2. Denaturation at 94° C for 1 min, primer annealing at 37° C for 1 min and primer extension at 72° C for 2 min, (step 2 was repeated 40 times)
3. Final extension step at 72° C for 7 min.

PCR amplification

PCR amplification was carried out with 25 ng of genomic DNA, optimized $MgCl_2$, optimized *Taq* DNA polymerase, 1X PCR buffer, optimized decamer primers and 200 μM of dNTP for optimization of component concentration. The volume was made up to 25 μl with sterile water. PCR tubes containing the above components were capped

and given a pulse spin to allow proper settling of reaction mixture. Thermocycling was carried out in a thermocycler (Eppendorf).

3.3.1.3 Optimization of conditions for ISSR

Forty ISSR primers (15-16mer oligos) were synthesized from Microsynth Gmbh, Switzerland and tested for PCR amplification with similar component concentrations used for optimization of PCR mixture (Table 3) similar to RAPD optimization. Amplifications were carried out with optimized $MgCl_2$), 200 µM dNTPs, 1X PCR buffer (0.1% Triton x-100, 2% formamide) optimized concentration of primer, optimized units Taq polymerase (MBI, Fermentas) and 25 ng of genomic DNA. Amplifications were carried out using a 96 thermal cycler programmed for 35 cycles as follows: an initial denatuartion was for 5 min at 94° C followed by 35 cycles of 1 min at 94° C, 1 min at 52° C, 2 min at 72° C and final extension for 7 min at 72° C.

3.3.1.4 Selection of polymorphic and reproducible markers

One hundred and twenty five decamer random primers of OPA to OPZ (Operon Technologies, USA) series and 40 ISSR primers were used to amplify genomic DNA of three randomly selected genotypes, which were included in the study. Those primers, which produced satisfactory amplification, polymorphic and reproducible band pattern, were selected for the individual genotypes analysis used for the diversity assessment.

3.3.1.5 Individual genotype analysis

Pre screened RAPD and ISSR primers were selected for the study of genetic relationship among 48 genotypes of *ber*.

Gel electrophoresis

Two µl of loading dye (6x) was added to each sample tube after completion of amplification. A 1.6% agarose gel was prepared with ethidium bromide (10 µl/100 ml) added to stain PCR amplified products. The contents of PCR tubes were loaded into the gel wells. Electrophoresis was carried out in 1x TAE buffer at 60 V for 3 hours, *i.e.,* till the bromophenol blue dye travelled less than 2/3 of the gel length. The resolved amplification products were visualized under UV light on a UV-Transilluminator and photographed using Gel-Documentation system (Gel Doc Mega, Biosystematica, UK).

3.3.1.6 Development of microsatellite markers

Microsatellites, also known as simple sequence repeats or SSRs, are a small array of tandemly arranged bases (one to six) spread throughout the genomes. Microsatellites as DNA markers are advantageous over many other markers as they are highly polymorphic, highly abundant, co-dominant inheritance, analytical simple and readily transferable (Sharma, 2005). Microsatellites are reported to be more variable than RFLPs or RAPDs, and have been widely utilized in plant genomic studies. The advantages of microsatellite over other types of genetic markers will become more important, and more obvious, when they are used to track desirable traits in large-scale breeding programs and as anchor points for map-based gene cloning strategies.

There are a number of ways to obtain microsatellite markers. Screening genomic libraries by hybridizing with SSR probes and sequencing the hybridized positive clones is

traditional but a laborious and costly approach. While screening the SSR-enriched, small-insert libraries can significantly reduce time and cost. In the present investigation, enriched-genomic library method was used to trace the microsatellites in *ber*. The genotype, Kala Gola was taken and DNA was isolated, purified and quantified. Simple hybrid capture method developed by Kandpal *et al.* (1994) with minor modifications was used to develop microsatellite primer.

3.4.1 Nebulization of genomic DNA and ligation of adaptors

A concentration of 10 µg of high quality and high molecular weight DNA was nebulized at 10 psi pressure for 10 seconds in 1 ml buffer containing 1.0 M Tris pH 8.0, 1.0 M $MgCl_2$ and 20% glycerol. The sheared DNA was precipitated, dissolved in water end polished to make ends of fragments blunt. This end polishing was done with 2 units of Klenow fragment (MBI Fermentas, USA) at 37° C for 10 min. 1µg adaptor oligos (OGA with sequence 5′-CTC TTG CTT ACG CGT GGA -3′ and OGB with sequence 5′-PO_4 -TAG TCC ACG CGT AAG CAA GAG -3′) were ligated to the 0.5 µg of blunt end fragment using 3 unit of T4 DNA ligase (MBI Fermentas, USA) and incubated overnight at 16°C as per manufacturer's instructions. The ligated fragments were purified by passing whole reaction mixture through Qiaquick columns (Qiagen Inc., Valencia, CA, USA). The volume of the recovered purified product was made to 50 µl with autoclaved distilled water. Of the recovered purified ligated product, 1.0 µl was used as template in PCR with adaptor specific primers using the following condition:

1 cycle at	94^0C	-	4 min
25 cycle at	94^0C	-	1 min
	55^0C	-	1 min
	72^0C	-	2 min
1 cycle at	72^0C	-	10 min

The PCR aliquot was subjected to 2% agarose gel electrophoresis to confirm the presence of smear. Smear between size range 400-1000 bps was eluted from the gel and the fragments were purified for further use.

3.4.2 Hybridization to biotinlyated oligos

PCR product (500 ng) eluted from gel was hybridized to 1 µg 5′-biotinylated oligos of $[GA]_{20}$, $[CA]_{20}$, $[AGA]_{15}$, $[ACA]_{15}$, $[CAT]_{15}$ and $[GATA]_{20}$ each. Before hybridization, the PCR product was denatured by heating it at 94° C for 10 min and kept on ice. Sodium phosphate buffer (pH 7.4) at 0.5M concentration along with the biotinylated oligo and 0.5% of sodium dodecyl sulphate (SDS) was added to the denatured PCR product and incubated at 50° C in a volume of 100 µl.

3.4.3 Enrichment of library for microsatellites

A 0.6 ml tube of straptavidin-coated beads (New England Biolabs Inc.) was washed three times with 0.6X SSC (Saline Sodium Citrate) buffer. Each time, magnetic particle concentrator was used to remove the solution by pipetting, while the beads in

contact with the magnetic concentrator adhere to the side of the tube. To the washed beads, the entire hybridization mixture was added and incubated at room temperature for 30min minutes. For enrichment, a series of washes are carried out at increasing stringency to wash away the un-hybridized DNA at every step leaving behind beads with bound DNA. The incubation condition and concentration of wash solution used was as detailed below

1.	6 x SSC buffer	- 15 min at	room temperature
2.	6 x SSC buffer	- 15 min at	65° C
3.	6 x SSC /0.1 % SDS buffer	- 15 min at	65° C (twice)

Finally, DNA was eluted in 50 µl of TE and was purified using Qiaquick columns and amplified in PCR reactions as described in section 3.4.1. The PCR product was quantified and used to set up ligation for cloning in pGEM-T vector.

3.4.4 Ligation and Cloning

The microsatellites enriched fragments were cloned into pGEM-T Essay vector following the manufactories protocol. The ratio 3:1 (insert: vector) was used for ligation. The ligated mixture was purified using ethanol precipitation and was then electoporated into DH10*B* electrocompetant cells. Electroporation was carried out in compatible cuvette in electroporator (Biorad). The transformed cells culture was incubated in growth culture medium (LB-Luria Broath) at 37° C (250 rpm for 1 hr). The culture was plated on culture plate containing LB-agar medium + amplicillin (100 µg/µl) with 50 µl of 200

mM IPTG and 20 µl of 50 mg X-gal. The plate was allowed to dry before plating the culture. A volume of 10 µl of culture was evenly spread over the media and then allowed to grow overnight at 37° C.

3.4.5 Selection of positive/transformed clones

White/transformed colonies were picked using toothpicks and inoculated in each single well of 96 well GM-plates (Glycerol Mount) for storage and also for 96 well deep well plates (for plasmid isolation). The plates were covered with air-pore tape sheets for proper aeration and kept for growth of culture in incubator-shaker overnight at 37°C at 250 rpm overnight.

After incubation, plates were removed from the incubator shaker and were processed separately for respective used. The 96 well GM plate was labeled properly sealed with aluminum foil sheets and stored at -80° C. The deep well plates were used for plasmid isolation using REAL kit (Qiagen), as per the manufacture's instructions. The isolated plasmid was stored at -20° C.

3.4.6 Pooling and amplification of plasmid DNA

The pooling of plasmid DNA from each clone was done in 3 steps:

(i) Initially, there were 12 × 96 plasmid DNA samples in 12 deep well plates (96 samples in each plate)

(ii) Pooling of 10 µl of all wells of column 1 of plate to A1, column 2 of plate to A2 and so on, to get pooled samples of 12 × 96 clones (12 × 12 wells).

(iii) From the earlier pooled samples, 10 µl from each well of a row was taken and pooled to make final 12 samples. The new pool represent samples of one plate in one well.

Then three step PCR was performed as follows.

(i) PCR from 12 wells- using primer of adapter sequences (microsatellite regions primers) for which screening is be done. Thus those plates with putative microsatellite region insert were identified.

(ii) Second step of PCR screening was done to identifying the row of the plates, which were selected in step using the pooled samples plate.

(iii) In the third step, those rows, which tested positive, were used for PCR of individual clone samples. The results by this PCR analysis, helped in exact identification of positive clones with the microsatellite region for which screening was done.

The PCR conditions used for screening of clones is as mentioned below:

DNA	-	30 mg
Buffer	-	1 x
$MgCl_2$	-	2.5 mM

dNTPs	-	150 mM
Primers (p1+P2)	-	20 pm each
Taq DNA polymerase	-	1 unit

Amplification condition was as follows

1 cycle	94° C	-	5 min
30 cycle	94° C	-	1 min
	50° C	-	1 min
	72° C	-	1 min
1 cycle	72° C	-	10 min

The size fractionation was done on 1.5% agarose gel and positive clones were identified.

3.4.7 Sequencing of selected clones

The positive clones were sequenced at capillary based-MEGABace 1000DNA sequencer (Amersham Biosciences). The sequencer sequence the DNA based on Sanger's Dideoxy chain termination method. Before sequencing, PCR was performed with 100ng DNA, 5.0 pmol universal M13 flouroscent primer and 4 µl of dNTPs with the PCR conditions of 35 cycles at 95° C for 10 sec, 50° C for 10 sec and 60° C for 2 min. The PCR products were purified and sequenced with single-pass sequencing. After sequencing quality of the sequences were checked with the 'Molecular Dynamics' software that determine the quality based on the peak height and the width of the sequences on the chromatogram.

3.4.8 SSR identification and primer development

The sequenced data was searched for microsatellite repeats with SSRIT (simple sequence repeat identification tool) for different repeat motifs (dimer, trimer, tetramer and pentamer) with an aim to detect repeat region of at least 10 nucleotides using dimer ≥5 repeats, trimer ≥4 repeats, tetramer ≥3repeats and pentamer≥2 repeats. The identified SSRs with 200-250 nucleotides on either side of the sequenced clones were used as frame sequence. For primer development the SSR primer discovery tool was used (Love *et al.*, 2004) with the criteria of melting temperatures between 50° C and 70° C (optimum 55°C), GC content of <70% and primer length of 18-23 nucleotide with a low chance of dinucleotide or hair-loop formation. The range for PCR product length was set to be between 100 and 500 bp.

3.4.9 Validation of microsatellite primers

Validation of developed microsatellite/ SSR primers was done for the amplificability and polymorphism to asses their suitability for fingerprinting and genetic relationship studies. The validation study was done using 48 *ber* genotypes used.

3.5 Statistical Analysis

3.5.1 Scoring and diversity analysis

Each band was treated as one RAPD marker. Scoring of bands was done from the photographs. Homology of bands was based on their migration distance in the gel. The presence of a band was scored as "1", absence of a band as "0" and missing datum was denoted by "9".

Gel scoring for 'qualitative' type of data

Presence/absence of a band in j^{th} and i^{th} taxa	J	+	-
I	+	a	B
	-	c	D

Where, m = a + d (number of matches)

u = b + c (number of unmatches)

n = u + m (total sample size)

The Jaccard's similarity coefficient (J) was used to calculate the similarity between pair's accessions (Jaccard, 1908) as: J = a/n-d

The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendogram was generated. These computations were performed using the programme NTSYS-PC Ver. 1.7 (Rohlf, 1993).

3.5.2 Resolving power (Rp)

Resolving power was used to identify the primers that would distinguish the genotypes most efficiently (Prevost and Wilkinson, 1999). Resolving Power (R_P) of a primer was calculated as the sum of "band informativeness" of all bands produced by a primer. Resolving power is based on distribution of alleles within the sampled genotypes. For most molecular fingerprinting systems, the division of genotypes into two groups is based on the presence or absence of a band at a particular position. Ideally, each band position would therefore be present in half of the genotypes and absent from the other half. The value of a particular band position can be measured by its similarity to the optimal conditions (50% of genotypes containing the band). This 'band informativeness' (I_b) can be represented into a 0-1 scale by the formula: $I_b = 1- |2 \times (0.5-p)|$, where p being the proportion of the 48 genotypes containing the band (Prevost and Wilkinson, 1999). The I_b value was calculated for all the informative bands for ISSR and RAPD primers that were scored in the study. If all bands were optimally informative, then the most useful primer or techniques would be those that generate the maximum band positions. Given that bands can be weighted according to their similarity to optimal informativeness, the ability of a primer or technique to distinguish between large numbers of genotypes, *i.e.* Resolving Power of the primer (Rp) could be represented by the sum of these adjusted values. This can be described as: $Rp=\sum Ib$. The Rp of RAPD and ISSR primers were determined in this way.

Band informativeness (I_{bi}) = $1 - [2(I_{0.5}-p_iI)]$

Where, 'p_i' is the proportion of genotypes containing the i^{th} band and I_{bi} is the informativeness of the i^{th} band.

Resolving power of the primer (R_p) is represented as $R_p = \Sigma I_b$. The information content of each primer was also determined according to the Marker Index (MI) given by Powell *et al.* (1997). Marker index is the product of expected heterozygosity/gene diversity (GD) and effective multiplex ratio (EMR).

Expected heterozygosity/gene diversity = $P\ (1-\Sigma p_i^2) / n$

Where, 'p_i' is the frequency of the i^{th} allele and 'n' is the number of genotypes.

EMR = Number of polymorphic products amplified per reaction.

3.5.3 Polymorphism information content (PIC)

The basic information about molecular markers that determines their application in genetic mapping was calculated for each marker by using the PIC (Lynch and Walsh, 1998). PIC expresses the discriminating power of the locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus, expressed as: $PIC = 1- \sum pi^2$, where p_i is the frequency of the *i*th (presence of band) allele. The ISSR and RAPD bands were scored as 1 (present) or 0 (absent) for each primer-genotype combination. For reproducibility of bands the study was carried out thrice on agarose gel. Only those bands, which showed amplification in all the three gels, were considered. The NTSYS-pc software package version 2.02 (Rohlf 1998) was used to calculate Jaccard's (1908) similarity coefficients among the genotypes and a dendrogram based on the similarity co-efficient was generated using Unweighted Paired

Group Method Arithmetic Mean (UPGMA) method. Most informative primers were selected based on the extent of polymorphism and resolving power of each primer was calculated by method suggested by Prevost *et al.* (1999). Polymorphism information content (PIC) was used to identify the primers, which would distinguish the cultivars most efficiently (Botstein *et al.*, 1980).

Polymorphism information content (PIC) = $2 \times P_i \times Q_i$

Where, 'P_i' is the frequency of a band presence

'Q_i' is the frequency of a band absence.

Table 2: List of the ber accessions used for genetic relationship study

Code	Accessions	Species	Main areas of cultivation
1	Sindhura	*Z. mauritiana* Lam.	Haryana
2	Seb	*Z. mauritiana* Lam.	Haryana
3	Vilayati	*Z. mauritiana* Lam.	Haryana
4	Illaichi	*Z. mauritiana* Lam.	Uttar Pradesh
5	Gola	*Z. mauritiana* Lam.	Rajasthan / Haryana
6	Muria Mehrora	*Z. mauritiana* Lam.	Uttar Pradesh
7	Katha	*Z. mauritiana* Lam.	Punjab
8	Thornless	*Z. mauritiana* Lam.	Uttar Pradesh
9	Sanauri	*Z. mauritiana* Lam.	Punjab
10	Banarasi Kadaka	*Z. mauritiana* Lam.	Uttar Pradesh
11	Chuahara	*Z. mauritiana* Lam.	Rajasthan/Punjab
12	Narma	*Z. mauritiana* Lam.	Delhi
13	Chonchal	*Z. mauritiana* Lam	Punjab
14	Z. *nummularia*	*Z. nummularia (Bum. f.) Willed*	Uttar Pradesh
15	Bagwadi	*Z. mauritiana* Lam	Rajasthan
16	Pownda	*Z. mauritiana* Lam.	Punjab
17	Sindhura Narwal	*Z. mauritiana* Lam.	Haryana
18	Kala Gola	*Z. mauritiana* Lam.	Haryana
19	Tass Bataso	*Z. mauritiana* Lam.	Uttar Pradesh
20	Tikadi	*Z. mauritiana* Lam.	Rajasthan
21	Dandan	*Z. mauritiana* Lam.	Rajasthan
22	Umran	*Z. mauritiana* Lam.	Punjab
23	Lakhan	*Z. mauritiana* Lam.	Punjab
24	Katha Bombay	*Z. mauritiana* Lam.	Gujarat

25	Bawal Selection -2	*Z. mauritiana* Lam.	Haryana
26	Pathan	*Z. mauritiana* Lam.	Punjab
27	Akhrota	*Z. mauritiana* Lam.	Punjab/Haryana
28	Rohtak Safeda	*Z. mauritiana* Lam.	Haryana
29	Sanauri-3	*Z. mauritiana* Lam.	Punjab
30	Jogia	*Z. mauritiana* Lam.	Uttar Pradesh
31	Desi Alwar	*Z. mauritiana* Lam.	Rajasthan
32	Nazuk	*Z. mauritiana* Lam.	Rajasthan
33	Hesang Tsaon	*Z. mauritiana* Lam.	Uttar Pradesh
34	No-A	*Z. mauritiana* Lam.	Haryana/Gujrat
35	Kathi	*Z. mauritiana* Lam.	Punjab/Gujrat/Rajasthan
36	Kaithali	*Z. mauritiana* Lam.	Haryana
37	Noki	*Z. mauritiana* Lam.	Punjab
38	Popular Gola	*Z. mauritiana* Lam.	Delhi
39	Kheera	*Z. mauritiana* Lam.	Uttar Pradesh
40	Sua	*Z. mauritiana* Lam.	Haryana
41	Katha Rajasthan	*Z. mauritiana* Lam.	Rajasthan
42	Reshmi	*Z. mauritiana* Lam.	Punjab
43	Wild collection	*Z. mauritiana* Lam.	Delhi
44	Govind Garh Selection	*Z. mauritiana* Lam.	Haryana
45	Kishmish	*Z. mauritiana* Lam.	Uttar Pradesh
46	Bawal Selection-1	*Z. mauritiana* Lam.	Bawal, Haryana
47	Zg-3	*Z. mauritiana* Lam.	Haryana
48	Illaichi Jhajhar	*Z. mauritiana* Lam.	Haryana

Chapter 4

RESULTS

The Indian *ber*, *Ziziphus mauritiana* Lam, a beautiful evergreen tree is an example of extremely drought-hardy species and is a dominant component of the natural vegetation of the Indian desert. The tree is endowed with a high degree of edaphic and climatic adaptability and can grow in different kind of soil types including the sub marginal lands. *Ziziphus* species are potentially very important in preventing soil erosion and desertification and has therefore, been planted in India and several other countries. However, indiscriminate felling of ber trees in the last 50 years has caused serious state of deterioration in their genetic resources. *Ziziphus* species are distributed over wide geographical and climatic condition that is indicative of its tremendous genetic diversity, which needs to be identified and catalogued. Although there exists a high level of morphological variability but the similar genotype is known by different names in different regions of cultivation, it is possibly due to influence of environment on morphology and therefore morphological appearances are not reliable measure of diversity among *ber* genotypes. Long juvenile phase of *Ziziphus* (up to 7 years) would make DNA markers an extremely useful tool for the identification of cultivars during propagation and growth. Molecular markers based on polymerase chain reaction (PCR) method offer several advantages over the sole use of conventional morphological markers. The PCR technology has led to the development of two simple and quick techniques *viz.*, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR). The former detects nucleotide sequence polymorphisms with a single primer of arbitrary nucleotide sequence and the latter permits detection of polymorphisms in inter-microsatellite loci with primers designed from dinucleotide or trinucleotide simple sequence repeats. Both the techniques are inexpensive and readily adaptable for routine germplasm fingerprinting

and evaluation of genetic relationship between accessions or genotypes and construction of genetic linkage maps.

The genotypes of *ber* used in the present study represent only a subset of the existing natural variation in the species (*Ziziphus mauritiana*) that are grown in different geographical region of India). To design the future breeding strategies in this fruit crop, it is utmost important to assess the level of genetic variation. Therefore, the present study using RAPD and ISSR markers aimed to asses the genetic diversity among *ber* genotypes with the comparison of the two marker systems. The study also aimed to develop SSR markers and their validation. The significant results from this study are being described under following subheads:

Before, starting the optimization of PCR conditions DNA samples for all the 48 genotypes were quantified with gel assay method (Fig. 2) and spectrophotometer. The quantified DNA of all the samples was diluted to 10 ng/µl individually with autoclaved distilled water.

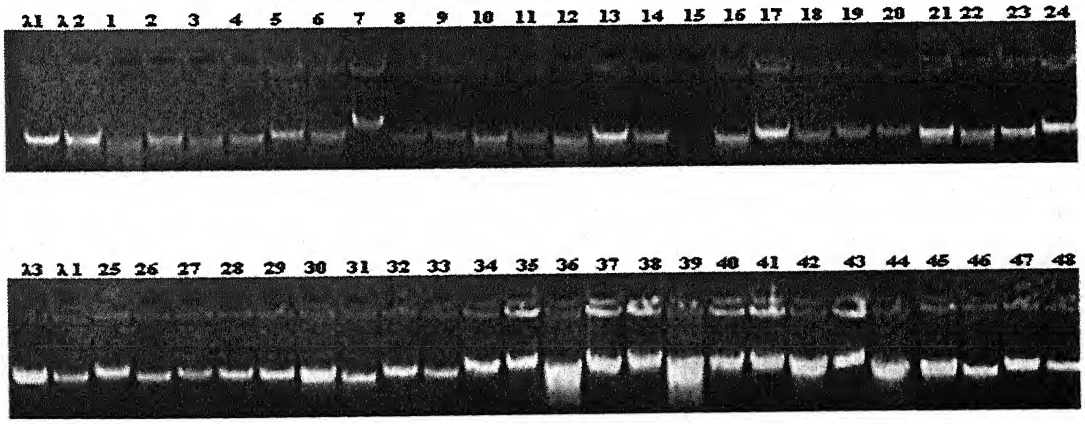

Fig. 2: Quantification of DNA extracted from 48 *ber* genotype (λ1:50 ng, λ 2:100 ng and λ3: 200 ng)

4.1 Development of DNA Profile Using RAPD Marker

A total of 120 RAPD primers (A, B, C, D, E and F series) from Operon Technology (Alameda, California, USA) were taken for the genetic relationship and diversity analysis among *ber* genotypes.

4.1.1 Optimization of PCR conditions

The PCR components for RAPD were optimized for *ber*. The dNTP, Taq DNA polymerase and $MgCl_2$ at concentrations of 200 µM, 1 unit and 2.0 mM, respectively provided good amplification. These components with the optimized concentration were taken for initial primer screening. The 4 bulks of DNA were made by pooling equal amount of DNA of 12 genotypes to make bulk 1, pooling DNA of another 12 genotypes to make bulk 2 and similarly for bulk 3 and 4 with different genotypes.

4.1.2 Band statistics

Out of the 120 RAPD primers, 46 primers were polymorphic. The 46 polymorphic primers amplified 368 fragments, of these 271 (74.15%) were polymorphic (Table 3). The bands were amplified within the size range of 150-5000 bp. The number of bands amplified ranged from 2 (OPF-9) to 17 (OPD-3), followed by 14 (OPD-7 and OPF-2), 12 (OPD-11 and OPF-11) and 11 (OPC-15, OPF-1 and OPF-8). The number of polymorphic bands ranged from 1 (OPF-9) to 15 (OPD-3), followed by 11 (OPF-2 and OPF-8), 10 (OPD-11) and 9 (OPC-15, OPD-4, OPF-11 and OPF-15). Nine primers provided 100% polymorphism (OPC-9, OPC-13, OPC-14, OPD-12, OPE-9, OPE-11, OPE-20, OPF-7 and OPF-8), followed by OPD-4 and OPF-15 (90.0%), OPE-6 (88.89%) and OPD-3 (88.24). The lowest polymorphism was observed for the primer OPC-11 (30.0%). The bands statistics amplified for individual primer is described hereunder:

OPC-2, OPC-3, OPC-4 and OPC-6

The primer OPC-2 amplified 8 bands within the size range of 200-700 bp, of which 3 (37.50%) were polymorphic. The resolving power and PIC of the primer was 1.04 and 0.26, respectively.

The primer OPC-3 amplified 9 bands within the size range of 200-900 bp, of which 6 (66.67%) were polymorphic. The resolving power and PIC of the primer was 3.29 and 0.54, respectively.

The primer OPC-4 amplified 6 bands within the size range of 180-350 bp, of which 4 (66.67%) were polymorphic. The resolving power and PIC of the primer was 3.00 and 0.44, respectively.

The primer OPC-6 amplified 8 bands within the size range of 190-800 bp, of which 7 (87.50%) were polymorphic. The resolving power and PIC of the primer was 3.67 and 0.51, respectively.

OPC-7, OPC-8, OPC-9, OPC-10

The primer OPC-7 amplified 7 bands within the size range of 500-1500 bp, of which 5 (71.43%) were polymorphic. The resolving power and PIC of the primer was 2.33 and 0.40, respectively.

The primer OPC-8 amplified 7 bands within the size range of 250-1500 bp, of which 4 (57.14%) were polymorphic. The resolving power and PIC of the primer was 3.08 and 0.47, respectively.

The primer OPC-9 amplified 3 bands within the size range of 700-1500 bp, of which 3 (100.00%) were polymorphic. The resolving power and PIC of the primer was 0.58 and 0.39, respectively.

The primer OPC-10 amplified 6 bands within the size range of 450-2000 bp, of which 3 (50.00%) were polymorphic (Fig. 3). The resolving power and PIC of the primer was 2.21 and 0.34, respectively.

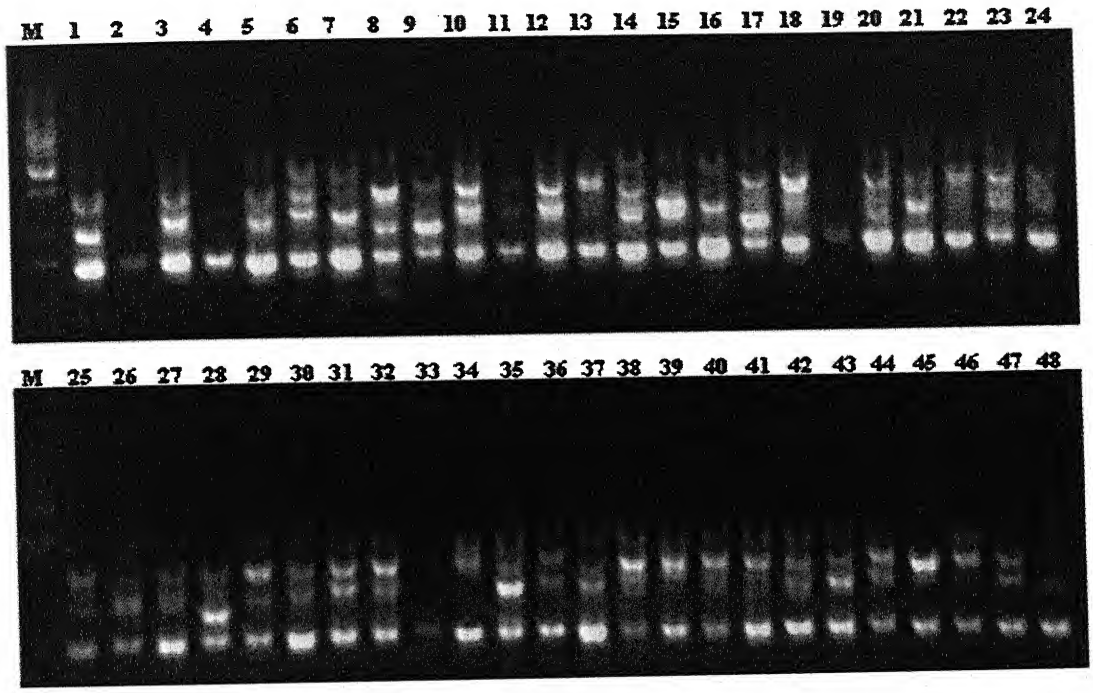

Fig. 3: RAPD profile of 48 *ber* genotype with OPC-10 (M: 100 bp)

OPC-11, OPC-13, OPC-14 and OPC-15

The primer OPC-11 amplified 10 bands within the size range of 450-2000 bp, of which 3 (30.00%) were polymorphic (Fig. 4). The resolving power and PIC of the primer was 1.21 and 0.28, respectively.

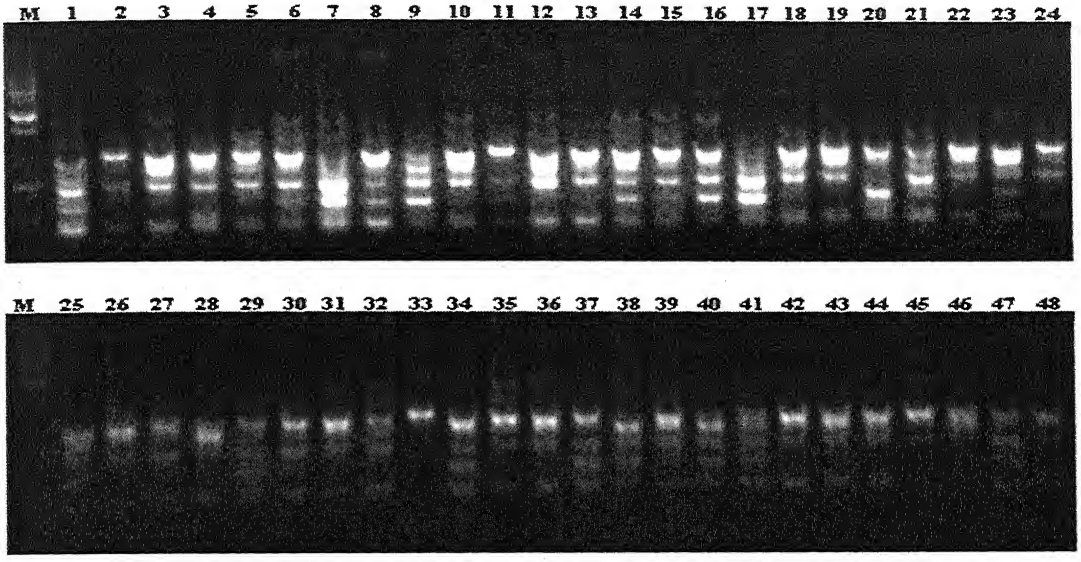

Fig. 4: RAPD profile of 48 *ber* genotype with OPC-11 (M: 100 bp)

The primer OPC-13 produced 100% polymorphism for 5 amplified bands within the size range of 500-3000 bp. The resolving power and PIC of the primer was 2.75 and 0.54, respectively.

The primer OPC-14 produced 100% polymorphism for 6 amplified bands within the size range of 500-1000 bp. The resolving power and PIC of the primer was 1.50 and 0.53, respectively.

The primer OPC-15 amplified 11 bands within the size range of 250-1500 bp, of which 9 (81.82%) were polymorphic. The resolving power and PIC of the primer was 5.92 and 0.71, respectively.

OPC-16, OPC-18, OPC-20 and OPD-3

The primer OPC-16 amplified 8 bands within the size range of 250-1500 bp, of which 4 (50.00%) were polymorphic (Fig. 5). The resolving power and PIC of the primer was 2.75 and 0.38, respectively.

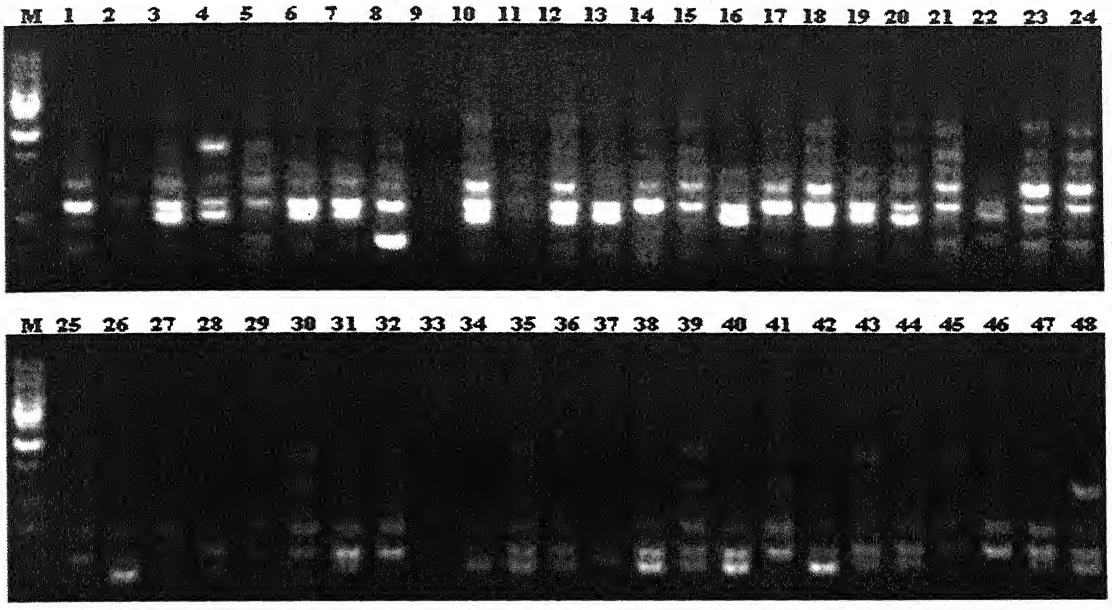

Fig. 5: RAPD profile of 48 *ber* genotype with OPC-16 (M: 100 bp)

The primer OPC-18 amplified 10 bands within the size range of 150-750 bp, of which 6 (60.00%) were polymorphic. The resolving power and PIC of the primer was 3.5 and 0.36, respectively.

The primer OPC-20 amplified 6 bands within the size range of 500-800 bp, of which 4 (66.67%) were polymorphic. The resolving power and PIC of the primer was 1.83 and 0.48, respectively.

The primer OPD-3 amplified 17 bands within the size range of 500-5000 bp, of which 15 (88.24%) were polymorphic (Fig. 6). The resolving power and PIC of the primer was 9.0 and 0.68, respectively.

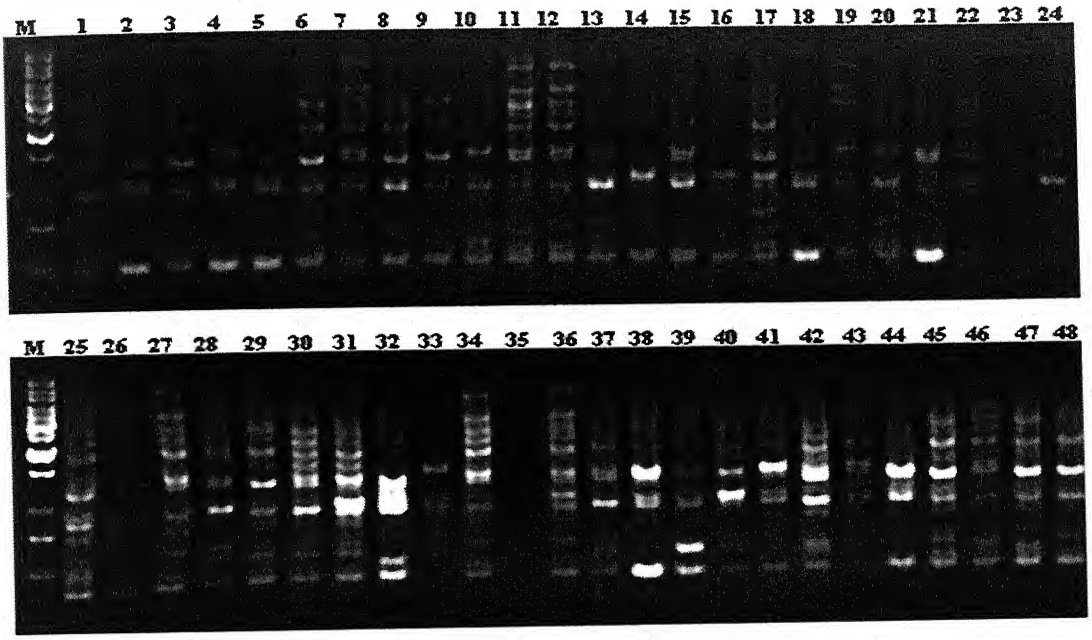

Fig. 6: RAPD profile of 48 *ber* genotype with OPD-3 (M: 100 bp)

OPD-4, OPD-7, OPD-8 and OPD-11

The primer OPD-4 amplified 10 bands within the size range of 450-1500 bp, of which 9 (90.00%) were polymorphic. The resolving power and PIC of the primer was 4.54 and 0.64, respectively.

The primer OPD-7 amplified 14 bands within the size range of 350-3000 bp, of which 6 (42.86%) were polymorphic (Fig 7). The resolving power and PIC of the primer was 3.33 and 0.32, respectively.

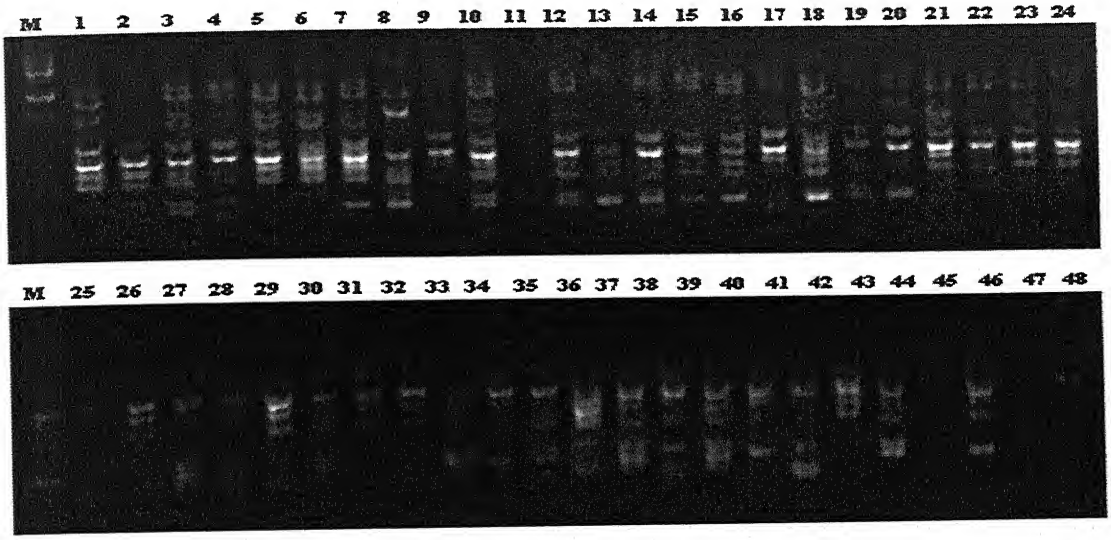

Fig. 7: RAPD profile of 48 *ber* genotype with OPD-7 (M: 100 bp)

The primer OPD-8 amplified 7 bands within the size range of 450-1100 bp, of which 6 (85.71%) were polymorphic. The resolving power and PIC of the primer was 3.08 and 0.55, respectively.

The primer OPD-11 amplified 12 bands within the size range of 250-1500 bp, of which 10 (83.33%) were polymorphic (Fig. 8). The resolving power and PIC of the primer was 5.42 and 0.52, respectively.

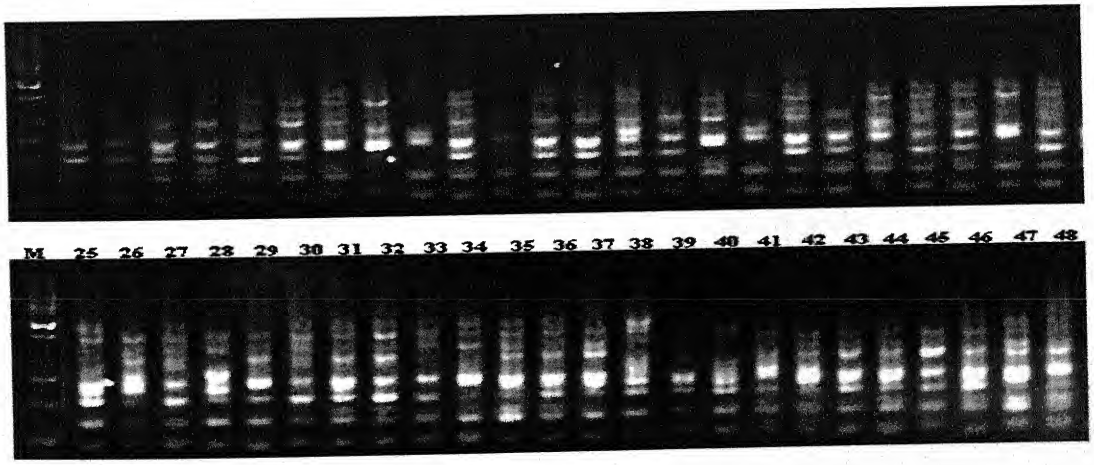

Fig. 8: RAPD profile of 48 *ber* genotype with OPD-11 (M: 100 bp)

OPD-12, OPD-20, OPE-2 and OPE-4

The primer OPD-12 produced 100% polymorphism for 6 amplified bands within the size range of 550-3000 bp. The resolving power and PIC of the primer was 1.96 and 0.60, respectively.

The primer OPD-20 amplified 7 bands within the size range of 200-450 bp, of which 2 (28.57%) were polymorphic. The resolving power and PIC of the primer was 0.92 and 0.11, respectively.

The primer OPE-2 amplified 10 bands within the size range of 400-3000 bp, of which 7 (70.00%) were polymorphic. The resolving power and PIC of the primer was 3.29 and 0.54, respectively.

The primer OPE-4 amplified 6 bands within the size range of 500-1800 bp, of which 3 (50.00%) were polymorphic. The resolving power and PIC of the primer was 0.46 and 0.34, respectively.

OPE-6, OPE-7, OPE-9 and OPE-11

The primer OPE-6 amplified 9 bands within the size range of 300-3000 bp, of which 8 (88.89%) were polymorphic. The resolving power and PIC of the primer was 5.54 and 0.58, respectively.

The primer OPE-7 amplified 3 bands within the size range of 450-750 bp, of which 2 (66.67%) were polymorphic. The resolving power and PIC of the primer was 0.92 and 0.25, respectively.

The primer OPE-9 produced 100% polymorphism for 4 amplified bands within the size range of 400-1031 bp. The resolving power and PIC of the primer was 2.83 and 0.82, respectively.

The primer OPE-11 produced 100% polymorphism for 5 amplified bands within the size range of 400-1250 bp. The resolving power and PIC of the primer was 1.92 and 0.44, respectively.

OPE-13, OPE-14, OPE-19 and OPE-20

The primer OPE-13 amplified 8 bands within the size range of 600-2000 bp, of which 7 (87.50%) were polymorphic. The resolving power and PIC of the primer was 3.67 and 0.72, respectively.

The primer OPE-14 amplified 8 bands within the size range of 200-1200 bp, of which 6 (75.00%) were polymorphic. The resolving power and PIC of the primer was 3.00 and 0.64, respectively.

The primer OPE-19 amplified 8 bands within the size range of 400-1800 bp, of which 6 (75.00%) were polymorphic. The resolving power and PIC of the primer was 4.33 and 0.49, respectively.

The primer OPE-20 produced 100% polymorphism for 8 amplified bands within the size range of 300-1500 bp. The resolving power and PIC of the primer was 3.75 and 0.45, respectively.

OPF-1, OPF-2, OPF-3 and OPF-4

The primer OPF-1 amplified 11 bands within the size range of 400-3000 bp, of which 6 (54.55%) were polymorphic. The resolving power and PIC of the primer was 4.67 and 0.41, respectively.

The primer OPF-2 amplified 14 bands within the size range of 250-3000 bp, of which 11 (78.57%) were polymorphic. The resolving power and PIC of the primer was 3.75 and 0.71, respectively.

The primer OPF-3 amplified 9 bands within the size range of 500-3000 bp, of which 7 (77.78%) were polymorphic (Fig. 9). The resolving power and PIC of the primer was 4.04 and 0.48, respectively.

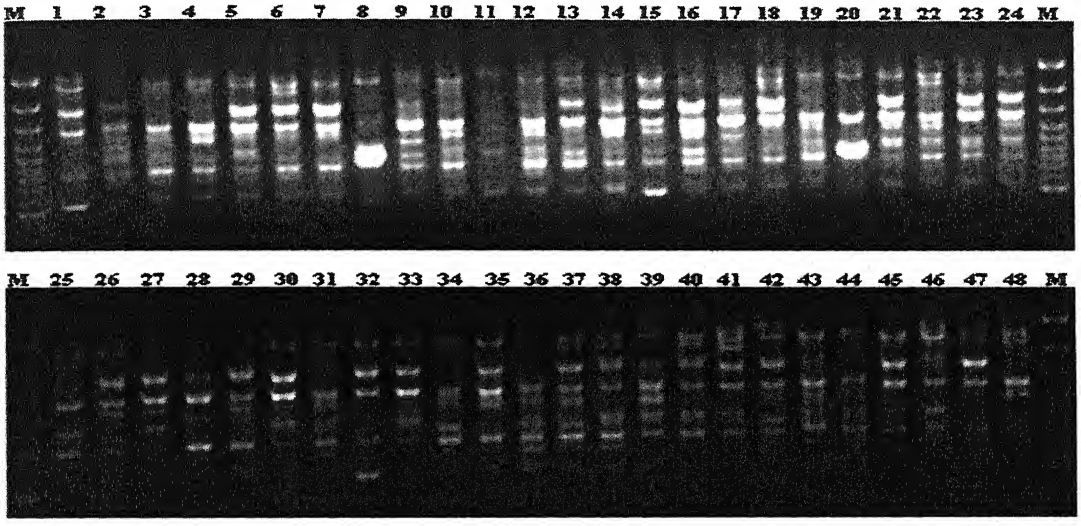

Fig. 9: RAPD profile of 48 *ber* genotype with OPF-3 (M: 100 bp)

The primer OPF-4 amplified 7 bands within the size range of 600-1400 bp, of which 5 (71.43%) were polymorphic. The resolving power and PIC of the primer was 1.50 and 0.34, respectively.

OPF-6, OPF-7, OPF-8 and OPF-9

The primer OPF-6 amplified 7 bands within the size range of 400-3000 bp, of which 5 (71.43%) were polymorphic. The resolving power and PIC of the primer was 2.79 and 0.62, respectively.

The primer OPF-7 produced 100% polymorphism for 6 amplified bands within the size range of 900-3000 bp. The resolving power and PIC of the primer was 2.42 and 0.57, respectively.

The primer OPF-8 produced 100% polymorphism for 11 amplified bands within the size range of 200-3000 bp. The resolving power and PIC of the primer was 5.75 and 0.71, respectively.

The primer OPF-9 amplified 2 bands within the size range of 500-900 bp, of which 1 (50.00%) were polymorphic. The resolving power and PIC of the primer was 0.96 and 0.39, respectively.

OPF-10, OPF-11, OPF-12 and OPF-13

The primer OPF-10 amplified 6 bands within the size range of 600-2000 bp, of which 4 (66.67%) were polymorphic. The resolving power and PIC of the primer was 2.13 and 0.56, respectively.

The primer OPF-11 amplified 12 bands within the size range of 400-1250 bp, of which 9 (75.00%) were polymorphic. The resolving power and PIC of the primer was 3.83 and 0.61, respectively.

The primer OPF-12 amplified 9 bands within the size range of 400-2000 bp, of which 6 (66.67%) were polymorphic. The resolving power and PIC of the primer was 2.21 and 0.54, respectively.

The primer OPF-13 amplified 5 bands within the size range of 400-2000 bp, of which 4 (80.00%) were polymorphic. The resolving power and PIC of the primer was 1.79 and 0.29, respectively.

OPF-15 and OPF-16

The primer OPF-15 amplified 10 bands within the size range of 600-3000 bp, of which 9 (90.00%) were polymorphic. The resolving power and PIC of the primer was 3.96 and 0.63, respectively.

The primer OPF-16 amplified 7 bands within the size range of 400-2000 bp, of which 5 (71.43%) were polymorphic. The resolving power and PIC of the primer was 4.17 and 0.52, respectively.

4.1.3 Resolving Power and polymorphism information content

The resolving power (Rp) of the 46 RAPD primers ranged from 0.46 (OPE- 4) to 9.00 (OPD-3) with an average of 3.06 per primer. RAPD primers, OPC-15, OPF-8, OPE-6 and OPD-11 posses high Rp values of 5.92, 5.75, 5.45 and 5.42, respectively (**Table 3**). The primers with high Rp values were able to distinguish more number of ber genotypes. Polymorphism information content (PIC) ranged from 0.11 (OPD-20) to 0.82 (OPE-9) with an average of value of 0.49 per primer. The other primers with high PIC values were OPE-13 (0.72), OPC-15, OPF-2 and OPF-8 (0.71), OPD-3 (0.68) and OPD-4 and OPE-14 (0.64).

4.1.4 Genetic relationship based on RAPD markers

Genetic similarity was estimated based on Jaccard's coefficient for RAPD markers. The genetic similarity between 48 accessions ranged from 26.3% (between Chhuhara and Thornless) to 78.9%. (Narma and Vilayati). Cluster analysis of the ber genotypes employing UPGMA led to the segregation of the accessions into two distinct groups (Fig. 10) – Cluster I and Cluster-II. In cluster-I, genotypes Seb and Chhuhara were grouped, whereas, cluster II comprised of remaining 45 genotypes of *Z. mauritiana* and one collection of *Z. nummularia*. In Cluster II, genotype Sindhura was ungrouped at the extreme top of the dendrogram. The remaining genotypes are separated from other genotypes at different similarity levels in successive branching. In cluster II genotypes were grouped into ten subclusters (II-a to II-j). The genotypes Thornless and Tikadi were grouped in subcluster II-a. In subcluster II-b six genotypes grouped together namely Rohtak Safeda, Sanauri-3, Bagwadi, Chonchal, Sindhura

Narwal and Sanauri. In subclusters II-c and II-d, three (Umran, Tass Bataso and Ilaichi) and two genotypes (No-A and Noki), respectively were grouped. In subcluster II-e six genotypes (Hesang Tsaon, Jogia, Akhrota, Katha Bombay, Dandan, Gola) were grouped, whereas, in subcluster II-f seven genotypes (Ilaichi Jhajhar, Zg-3, Kheera, Kishmish, Nazuk, Reshmi and Pathan) were grouped. In subcluster II-g Katha Rajasthan, Sua, Popular Gola and the second collection of *Z. nummularia* (from Delhi) were grouped.

The subcluster II-h comprised of three genotypes namely, Pownda, Katha and Mundia Mehrara. In cluster II-i Bawal Sel-2, Lakhan, and Kala Gola were grouped, whereas, in subcluster II-j remaining nine genotypes Bawal Sel-1, wild collection of *Z. mauritiana*, Kathi, Govindgarh Sel., Kaithali, Desi Alwar, Narma, Banarasi Karaka and Vilayati.

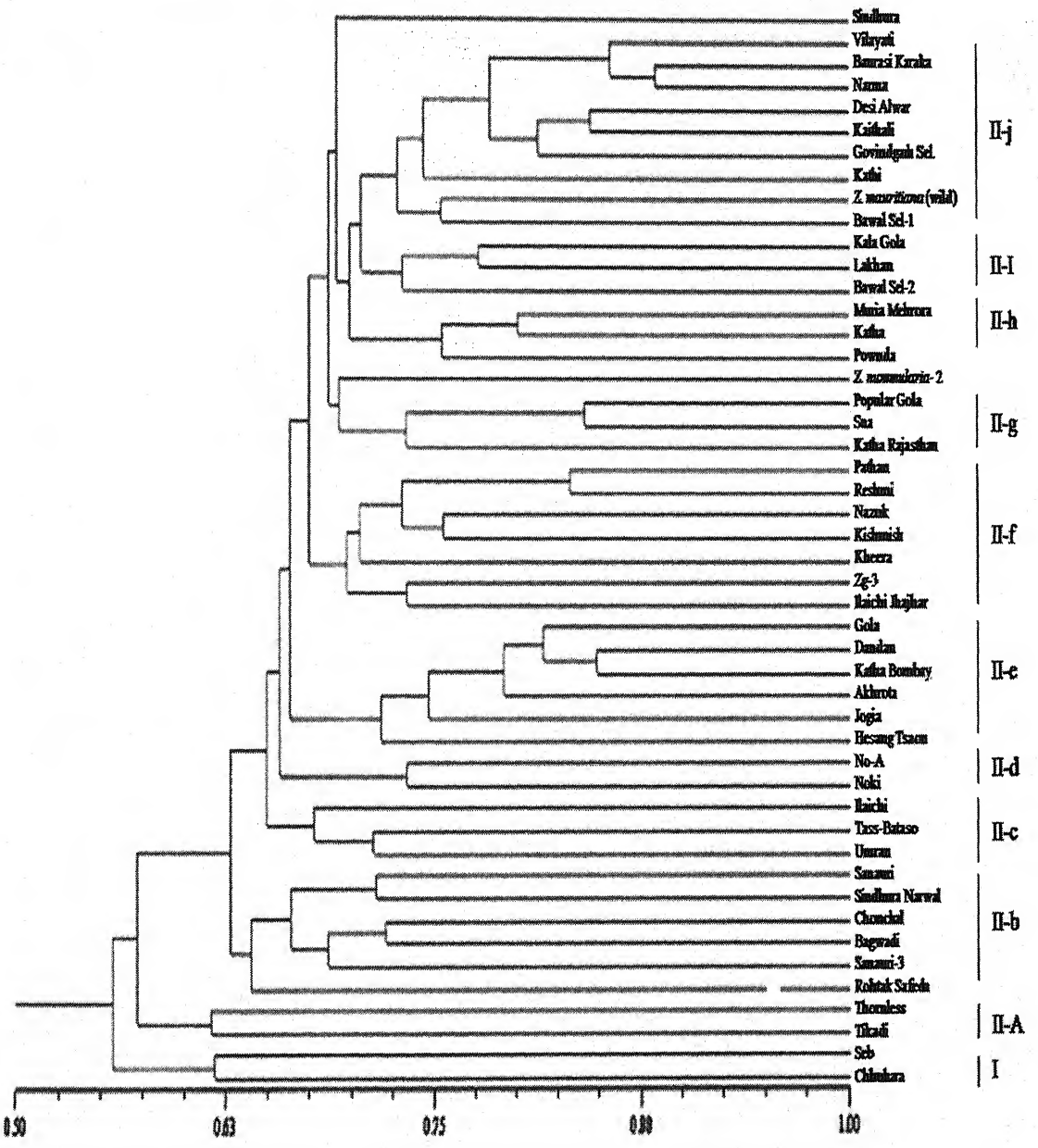

Fig. 10: Genetic relationship among 48 *ber* genotypes based on Jaccard's similarity coefficient for RAPD analysis

4.2 Development of DNA Profiles Using ISSR Marker

In the present study, a total of 40 ISSR primers from University of British Columbia (UBC) were taken for the genetic relationship and diversity analysis among 48 *ber* genotypes.

4.2.1 Optimization of PCR conditions

The PCR components for RAPD were optimized for *ber*. The dNTP, Taq DNA polymerase and $MgCl_2$ at concentrations of 200 µM, 1 unit and 1.5 mM respectively provided good amplification. These components with the optimized concentration were taken for initial primer screening. The 4 bulks of DNA were made by pooling equal amount of DNA of 12 genotypes to make bulk 1, pooling DNA of another 12 genotypes to make bulk 2 and similarly for bulk 3 and 4 with different genotypes.

4.2.2 Band statistics

Out of forty ISSR primers used, twenty one primers gave satisfactory amplification and band resolution and of these 18 were polymorphic. The 18 polymorphic primers generated 167 bands and the size of the amplification products ranged from 250 bp to 2500 bp (**Table 4**). The number of bands amplified ranged from 4 (UBC-856) to 12 (UBC-829 and UBC-894), followed by 11 (UBC-808 and UBC-809) and 10 (UBC-814, UBC-840, UBC-841, UBC-855 and UBC-900). The number of polymorphic bands ranged from 4 (UBC-856 and UBC-889) to 11 (UBC-894), followed by 10 in 6 primers viz., UBC-809, UBC-814, UBC-829, UBC-840, UBC-841 and UBC-900) and 9 in 5 primers viz., UBC-808, UBC-854, UBC-855, UBC-876, UBC-890. Nine primers provided 100% polymorphism (UBC-814, UBC-840, UBC-841, UBC-848, UBC-850, UBC-854, UBC-856, UBC-876 and UBC-890)

with average percentage polymorphism of 89.94%. The lowest polymorphism was observed for the primer UBC-889 (57.1%). The bands statistics amplified for individual primer is described hereunder:

UBC-808, UBC-809, UBC-814 and UBC-825

The primer UBC-808 amplified 11 bands within the size range of 400-1500 bp, of which 9 (81.8%) were polymorphic. The resolving power and PIC of the primer was 4.04 and 0.34, respectively.

The primer UBC-809 amplified 11 bands within the size range of 250-1200 bp, of which 10 (90.9%) were polymorphic (Fig 11). The resolving power and PIC of the primer was 4.75 and 0.37, respectively.

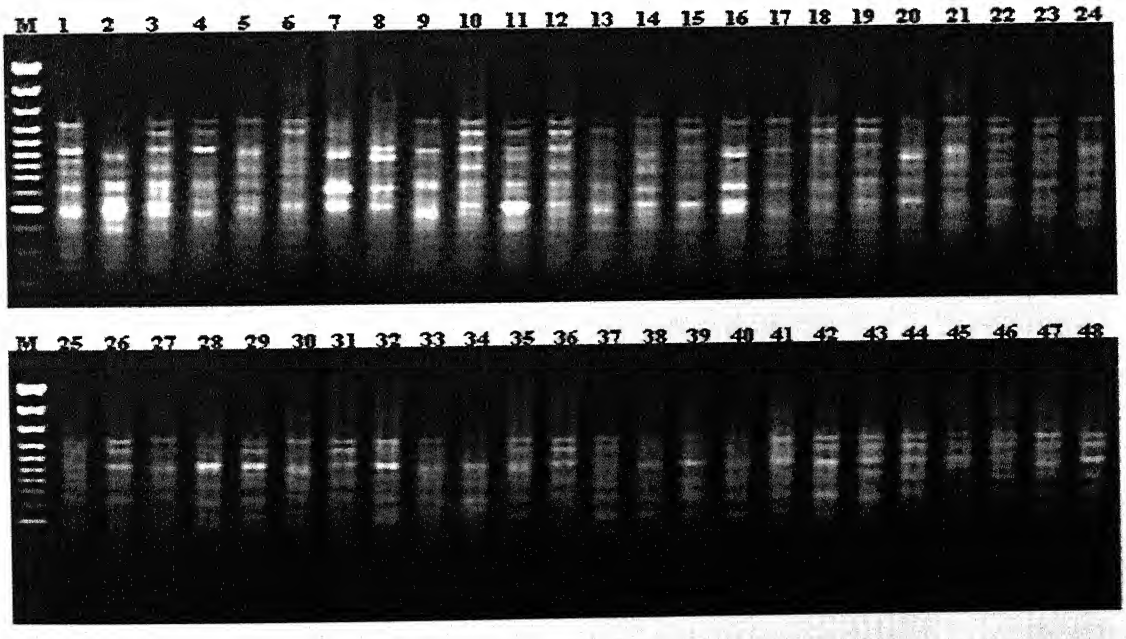

Fig. 11: ISSR profile of 48 *ber* genotype with UBC-809 (M: 100 bp)

The primer UBC-814 produced 100% polymorphism for 10 amplified bands within the size range of 400-1500 bp. The resolving power and PIC of the primer was 4.54 and 0.42, respectively.

The primer UBC-825 amplified 9 bands within the size range of 650-1500 bp, of which 6 (66.6%) were polymorphic. The resolving power and PIC of the primer was 2.34 and 0.23, respectively.

UBC-829, UBC-840, UBC-841, UBC-848

The primer UBC-829 amplified 12 bands within the size range of 400-1200 bp, of which 10 (83.3%) were polymorphic (Fig. 12). The resolving power and PIC of the primer was 4.21 and 0.51, respectively.

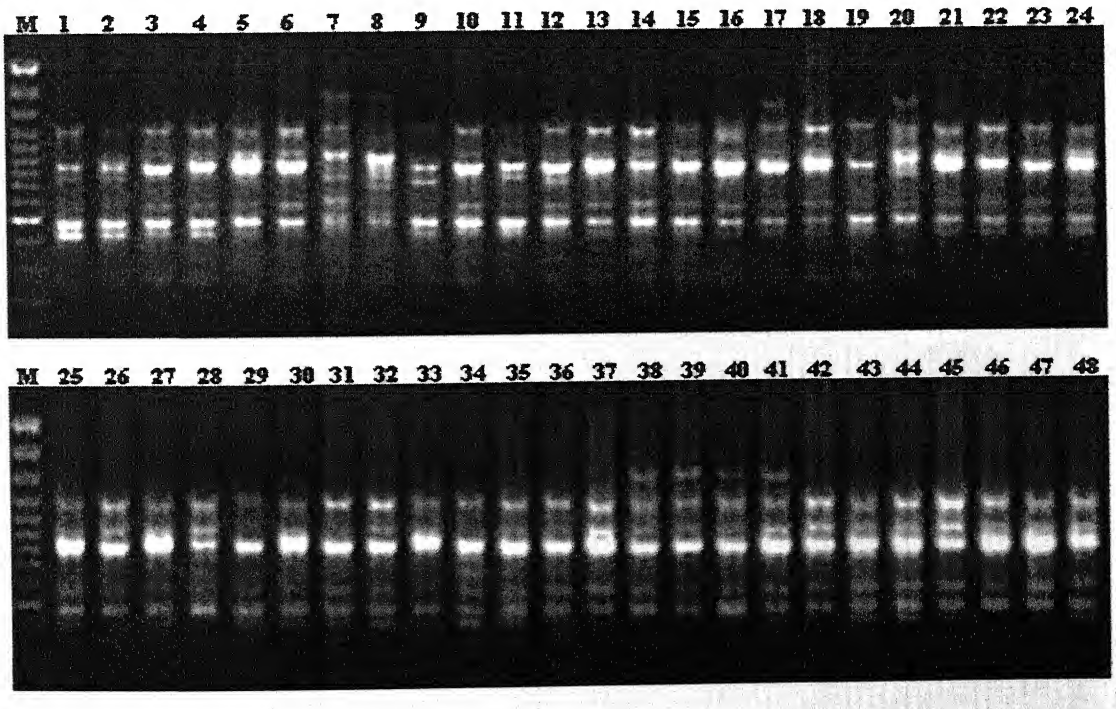

Fig. 12: ISSR profile of 48 *ber* genotype with UBC-829 (M: 100 bp)

The primer UBC-840 produced 100% polymorphism for 10 amplified bands within the size range of 700-2000 bp (Fig 13). The resolving power and PIC of the primer was 2.5 and 0.4, respectively.

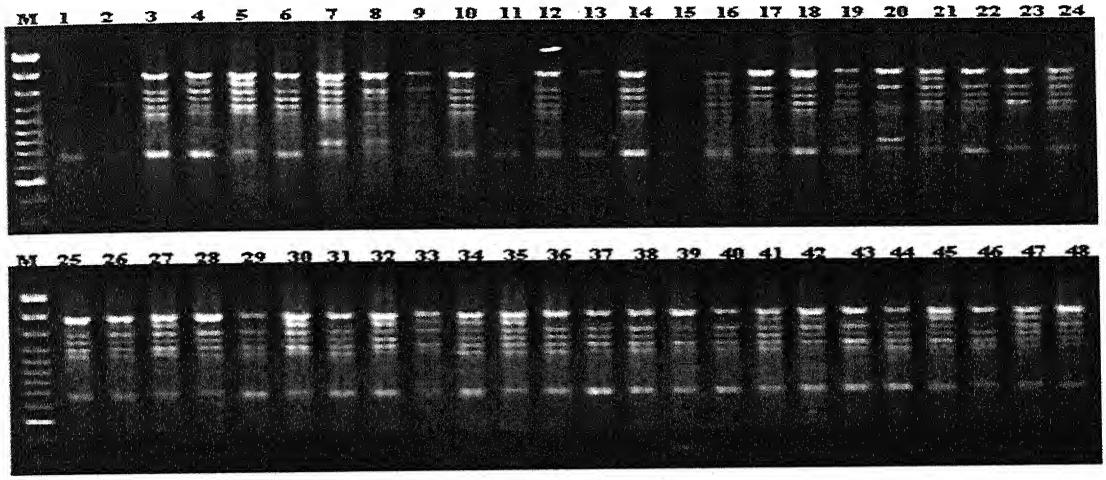

Fig. 13: ISSR profile of 48 *ber* genotype with UBC-840 (M: 100 bp)

The primer UBC-841produced 100% polymorphism for 10 amplified bands within the size range of 400-1500 bp (Fig 14). The resolving power and PIC of the primer was 5.29 and 0.53, respectively.

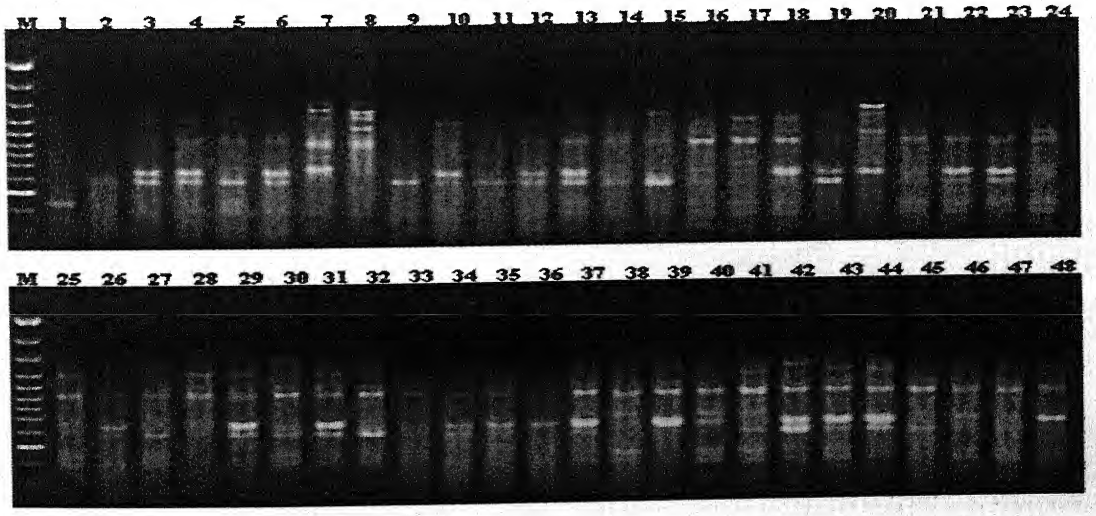

Fig. 14: ISSR profile of 48 *ber* genotype with UBC-841 (M: 100 bp)

The primer UBC-848 produced 100% polymorphism for 7 amplified bands within the size range of 500-2000 bp (Fig 15). The resolving power and PIC of the primer was 3.67 and 0.44,respectively.

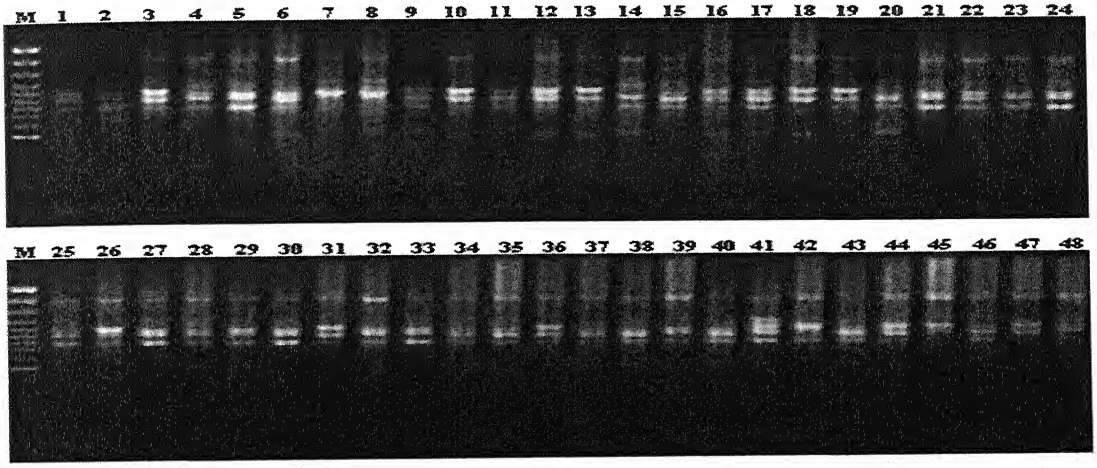

Fig. 15: ISSR profile of 48 *ber* genotype with UBC-848 (M: 100 bp)

UBC-850, UBC-854, UBC-855 and UBC-856

The primer UBC-850 produced 100% polymorphism for 8 amplified bands within the size range of 350-1031 bp (Fig 16). The resolving power and PIC of the primer was 4.71 and 0.64, respectively.

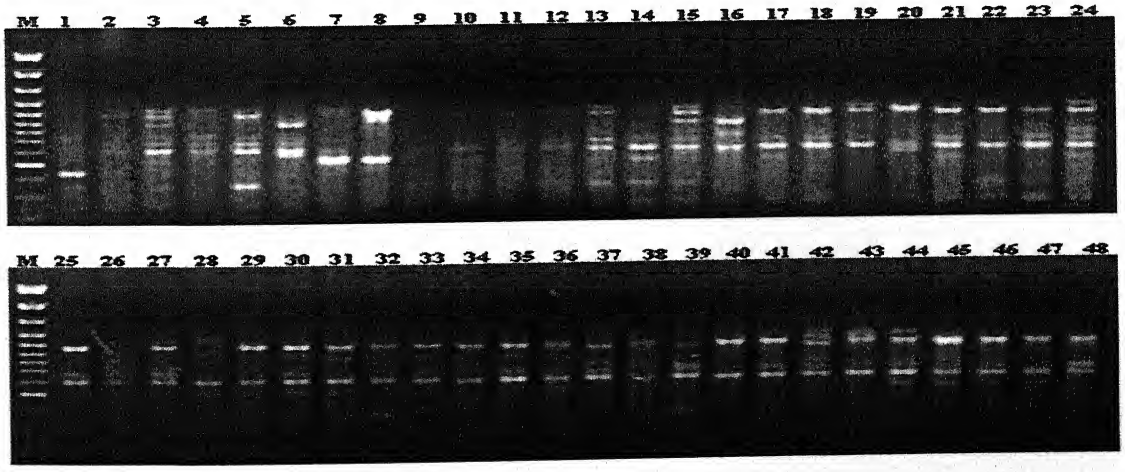

Fig. 16: ISSR profile of 48 *ber* genotype with UBC-850 (M: 100 bp)

The primer UBC-854 produced 100% polymorphism for 9 amplified bands within the size range of 700-1600 bp (Fig 17). The resolving power and PIC of the primer was 2.5 and 0.38, respectively.

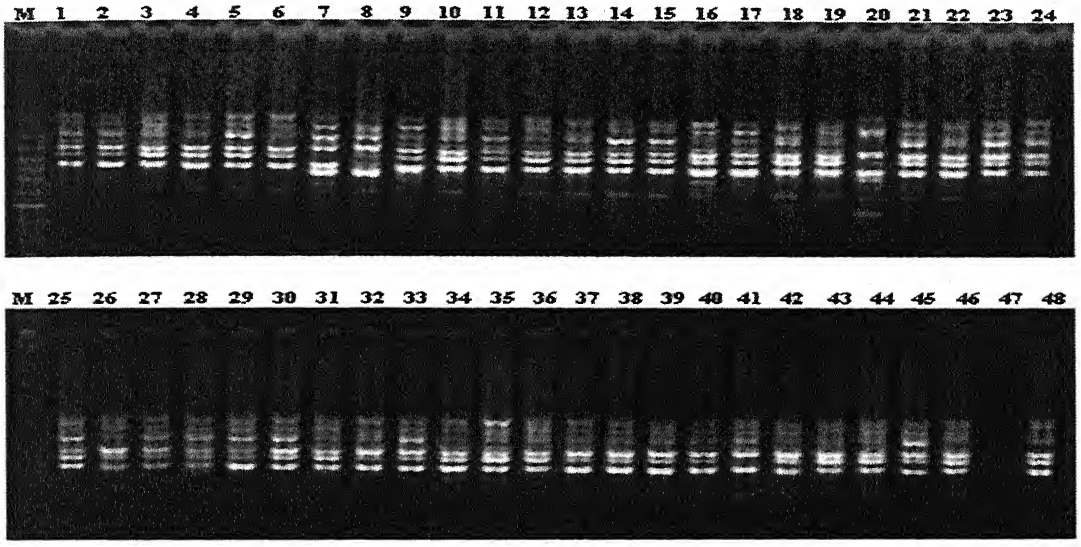

Fig. 17: ISSR profile of 48 *ber* genotype with UBC-854 (M: 100 bp ladder plus)

The primer UBC-855 amplified 10 bands within the size range of 500-1500 bp, of which 9 (90.0%) were polymorphic. The resolving power and PIC of the primer was 3.79 and 0.6, respectively.

The primer UBC-856 produced 100% polymorphism for 4 amplified bands within the size range of 800-2000 bp. The resolving power and PIC of the primer was 3.67 and 0.36, respectively.

UBC-876, UBC-880, UBC-889 and UBC-890

The primer UBC-876 produced 100% polymorphism for 9 amplified bands within the size range of 400-2000 bp. The resolving power and PIC of the primer was 3.71 and 0.36, respectively.

The primer UBC-880 amplified 9 bands within the size range of 350-1900 bp, of which 7 (77.8%) were polymorphic. The resolving power and PIC of the primer was 5.63 and 0.54, respectively.

The primer UBC-889 amplified 7 bands within the size range of 700-1200 bp, of which 4 (57.1%) were polymorphic. The resolving power and PIC of the primer was 2.04 and 0.24, respectively.

The primer UBC-890 produced 100% polymorphism for 9 amplified bands within the size range of 600-1500 bp. The resolving power and PIC of the primer was 1.00 and 0.56, respectively.

UBC-894 and UBC-900

The primer UBC-894 amplified 12 bands within the size range of 600-2000 bp, of which 11 (91.6%) were polymorphic. The resolving power and PIC of the primer was 5.17 and 0.43, respectively.

The primer UBC-900 amplified 10 bands within the size range of 500-2500 bp, of which 8 (80.0%) were polymorphic. The resolving power and PIC of the primer was 2.63 and 0.27, respectively.

4.2.3 Resolving Power and Polymorphism Information content

The resolving power (Rp) of the 18 ISSR primers ranged from 1.00 (UBC-890) to 5.63 (UBC-880) with an average of 3.68 per primer. ISSR primers viz., UBC-841, UBC-894, UBC-809 and UBC-850 posses high Rp values of 5.29, 5.17, 4.75 and 4.71, respectively (**Table 4**). The primers with high Rp values were able to distinguish more number of ber genotypes. Polymorphism information content (PIC) ranged from 0.23 (UBC-825) to 0.64

(UBC-850) with an average of value of 0.42 per primer. The other primers with high PIC values were UBC-855 (0.60), UBC-890 (0.56), UBC-880 (0.54) and UBC-841 (0.53).

4.2.4 Genetic diversity based on ISSR markers

Genetic similarity was estimated based on Jaccard's coefficient for ISSR markers. The genetic similarity between 48 accessions ranged from 43.1% (between Katha and Seb) to 90.3% (between Narma and Banarasi Karaka). Dendrogram based on genetic similarity values were constructed to reveal similarities between varieties. UPGMA based dendrogram (Fig. 18) grouped the genotypes in two main clusters. Cluster-I comprised of 2 genotypes, Katha and Thornless with the genetic similarity of 70.1% whereas, Cluster-II consisted of remaining 46 genotypes studied. Cluster-II separated genotypes Sindhura from any other genotype at the extreme top of the dendrogram. Cluster-II was further subdivided into 9 subclusters. The genotype Tikadi was separated at the extreme bottom of cluster-II. In subcluster II-a, two genotype were Pathan and Reshmi grouped together at 73.8% genetic similarity. In the subcluster II-b three genotypes, *Z. nummularia* (wild) and Bagwadi (with 73.1% similarity) were separated from Rohtak Safeda at an average similarity of 68.9%. In subcluster II-c, Umran and Lakhan shared 80.7% genetic similarity. Subcluster II-d consisted of genotypes, Chonchal, Kala Gola and Pownda. Subcluster II-e was the biggest subcluster containing 9 genotypes, Ilaichi, Elaichi Jhajhar, Gola, Dandan, Katha Bombay, Akhrota, Hesang Tsaon, Jogia and Sanuari-3. Subcluster II-f consisted of Nazuk, Noki, Popular Gola, Sua and Bawal Selection-1. Subcluster II-g consisted of Desi Alwar, Kathi, Kaithali, Govind Garh Selection, Zg-3 and Kheera. Subcluster II-h consisted of 4 genotypes, viz., Vilayati, Banarasi Karaka, Narma separated from Tass-Batasso at average genetic similarity of 78.9%. Subcluster II-i consisted of 5 genotypes, namely, Seb, Chhuhara, No-A,

Sanauri and Sindhura Narwal. Seven of the genotypes, Rohtak Safeda, Tikadi, Kishmish, Bawal Sel. 2, Katha Rajasthan, Muria Mehrara and Sindhura were ungrouped.

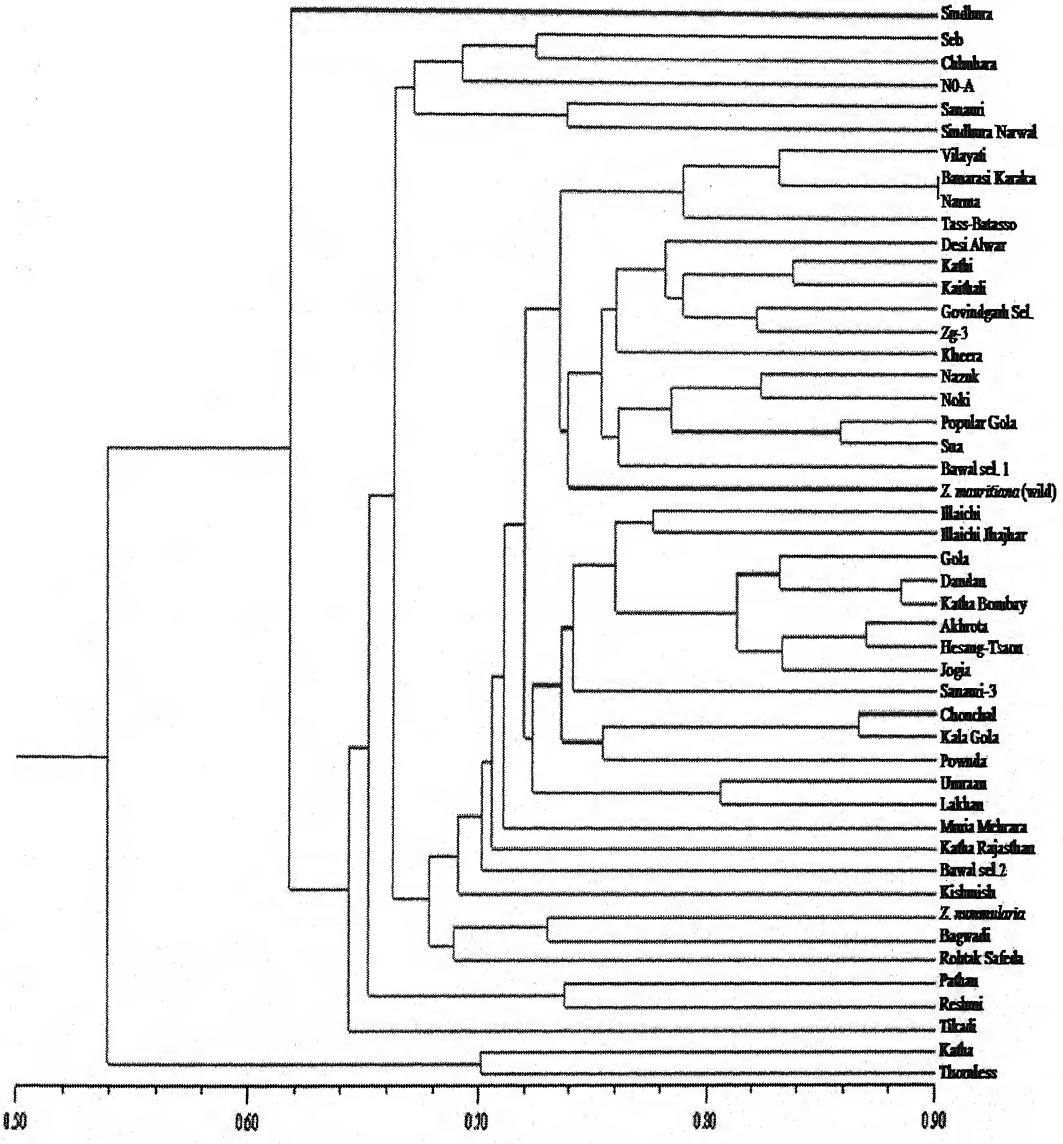

Fig. 18: Genetic relationship among 48 *ber* genotypes based on Jaccard's similarity coefficient for ISSR analysis

4.3 Development of New SSR Marker and Its Use

4.3.1 Microsatellite detection

The PCR product (500 ng) obtained from PCR amplification of nebulized and end-polished genomic DNA fragments with adaptor specific primers were eluted from gel and hybridized to 1µg 5′-biotinylated oligos of $[GA]_{20}$, $[CA]_{20}$, $[AGA]_{15}$, $[ACA]_{15}$, $[CAT]_{15}$ and $[GATA]_{20}$ each. Selected 192 of the positive colonies (Fig 19a and 19b) were then sequenced giving a total 307 sequences from M13 forward (171 sequences) and M13 reverse (136 sequences) primers. Out of the clones, sequenced 271 sequences (88.27%) were having repeat motifs. Of these 175 sequences (57.0%) contained sequence regions of high quality. Out of the 175 sequences, 78 (44.6%) were with more than 50% high quality sequence regions and 23 (13.1%) were more than 90% high quality sequence regions. The readable 175 sequences were traced using SSRIT and a total of 76 repeats were identified based on selection criteria of number of repeats i.e. ≥5 repeats for dimer, ≥4 repeats for trimer, ≥3 repeats for trimer and ≥2 repeats for pentamer so as to get a repeat region of at least 10 nucleotides. Out of the SSR identified 56 were based on pentamer repeats, followed by 14 dimer repeat, 11 tetramer and 2 trimer repeats (Appendix IV). The frequencies of di-, tri-, tetra- and pentamer repeats were calculated considering sequence complementarity.

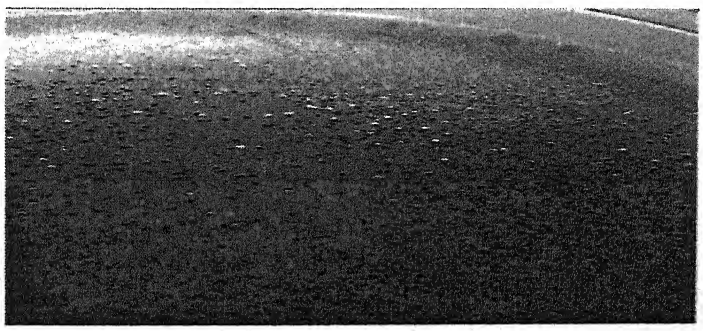

Fig. 19 a: Selection of positive colonies (blue) on X-gal IPTG medium

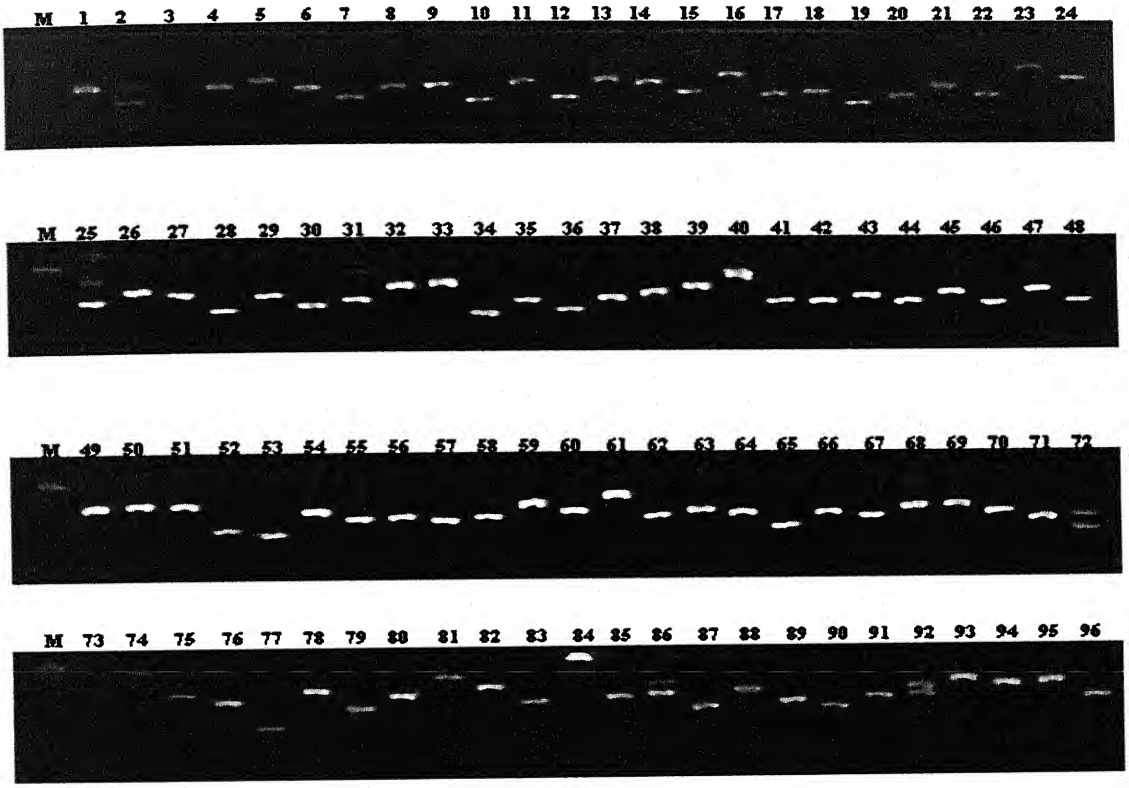

Fig. 19 b: Selection of positive clones for microsatellite enriched sequences

(M= 50 bp ladder, each array on the gel represent a single PCR product)

a) Dimer repeats identified

A total of 7 dimer repeats of 4 different repeat motifs were identified. The repeat motifs, CA/TG, AG/CT and TA were detected two times and GA/TC only once in the clones sequenced The largest repeat motif was $(CT)_{14 \text{ and } 8}$, followed by $(TA)_7$ and $(TA)_5$, $(GA)_5$ and two repeats of $(CA)_5$. Reducing the number of repeat motif in a stretch from 5 to 4, resulted in the high number of repeats for CT dimer (6) and TA (5) in the clones sequenced.

b) Trimer repeats identified

Two trimer repeats with repeat motif of $(GTA)_5$ and $(GAG)_4$ were identified. When the number of repeats for the individual repeat motif was reduced from 4 to 3, 14 different repeat

motifs were detected, of these AGA/TCT was detected 7 times, CTC/GAG (4 times), AAC/GTT, ATA/TAT, ATC/GAT, CAA/TTG, CCA/TGG and GAA/TTC (3 times) and GAC/GTC, ATG/CAT, CAC/GTG, GTA/TAC, TGT/ACA and TTA/TAA (2 times).

c) Tetramer repeats identified

Eleven tetramer repeats of 6 different repeat motifs were identified and all of these were repeated three times. These were TCTT/AAGA, TAAT/ATTA, TTAA, ATAC/GTAT, ACCA/TGGT and 6 repeats of at different sequence regions. When the number of repeats for the individual tetramer repeat motif was reduced from 3to 2, 81 different repeat motifs were detected, of these AATT and CGGC/GCCG was detected 22 times, ATGA/TCAT (16 times), ATAA/TTAT, CCAA/TTGG, CTTC/GAAG, TAAA/TTTA and TTCA/TGAA (14 times), AAGA/TCTT (13 times), ACAA/TTGT and ATCA/TGAT (13 times), AAGG/CCTT, AATC/GATT, ATTG/CAAT,CTAA/TTAG, GGAA/TTCC, TGGC/GCCA and TTTC/GAAA (10 times) and the remaining 63 repeat motifs were observed less than 10 times in the clones sequenced (one at 9 times, eight at 8 times, two at 7 times, nine at 6 times, one at 5 times, eighteen at 4 times and twenty four of the tetramers at 2 times of the clones sequenced). Out of tetramer repeats $(CGAC)_5$ was with the highest length of repeat motif.

d) Pentamer repeats identified

Fifty six pentamer repeats of 41 different repeat motifs were identified. Of these the maximum number of repeats were detected for the repeat CCAAG (4) followed by AATTA, ATCTA and AGGAA (3), GGCGG, TTGCG, TTGTT, AAAGA, AAGCT and AAGGT (2). The remaining 31 pentamer repeats were observed only once in the clones sequenced.

4.3.2 Validation of microsatellite markers

A total of 10 primer pairs based on the flanking sequences of identified repeats through SSRIT were synthesized to test the validity of these novel SSR primers for polymorphism and genetic relationship study. Amplification was successful for 8 out of 10 primers (80%). Of the 8 successfully amplified primers, 6 (75%) were polymorphic.

The high quality sequences were traced for microsatellite repeats and pair of primers (forward and reverse) developed from these sequences. A total of 10 primer pairs were developed and validated on 48 ber genotypes for polymorphism (**Table 5**). These primers were developed repetitive elements (7) and from non-repeat regions (3). Out of these, 71.4% primers from the repetitive elements (5) and 33.3% from non-repetitive elements were polymorphic. Out of the 10 primer pairs, 2 (Z_aksdc7 and Z_aksdc10) were not-scorable and provided smeared bands, therefore, not scored for polymorphism. Out of the 8 primers that generated scorable fragments, 6 were polymorphic. These 8 primers amplified 32 alleles, of which 17 (63.19%) were polymorphic. The level of polymorphism ranged between 20.0% and 100.0%. The Rp and PIC values for the microsatellite markers ranged between 0.42 to 3.42 and 0.09 to 0.77, respectively. Results obtained for amplified individual microsatellite primer is detailed hereunder:

Z_aksdc1

The primer Z_aksdc1 produced a single monomorphic band of 104 bp. The primer was developed from the $(TG)_5$ repeat in the clone >Zm01a03.y well A02.

Z_aksdc2

The primer Z_aksdc2 amplified 2 alleles with size of 167 bp and 324 bp One of these allele was uniquely amplified by the genotype Katha Bombay (Fig 20). The resolving power and

PIC of the primer was 0.42 and 0.5, respectively. The primer was developed from the $(GA)_5$ repeat in the clone >Zm01a05.z well.

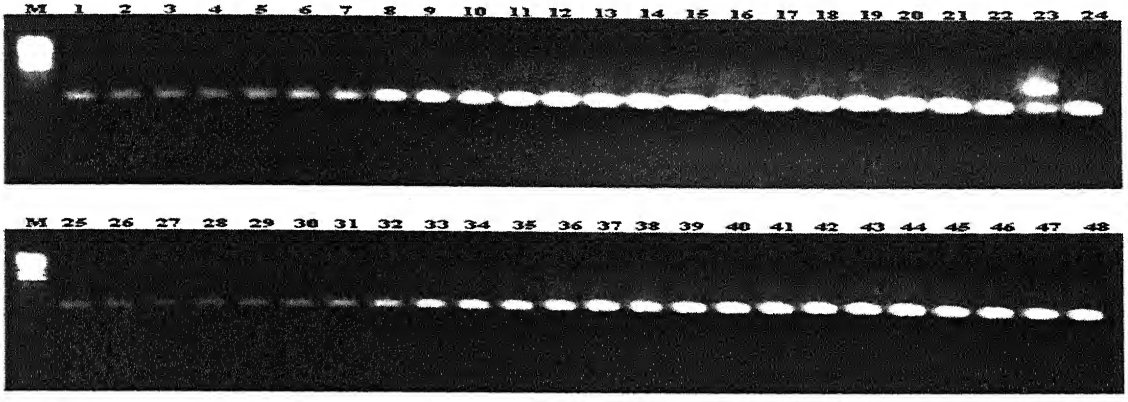

Fig. 20: SSR profile of 48 *ber* genotype with Z_aksdc2 (M: 100 bp ladder plus)

Z_aksdc3

The primer Z_aksdc5 amplified 5 alleles with size of 169 bp, 230 bp, 277 bp, 335 bp and 433 bp. One (20.0%) of these allele were polymorphic (Fig 4.3.2). The resolving power and PIC of the primer was 0.542 and 0.094, respectively. The primer was developed from the $(TA)_5$ repeat in the clone $(TA)_5$

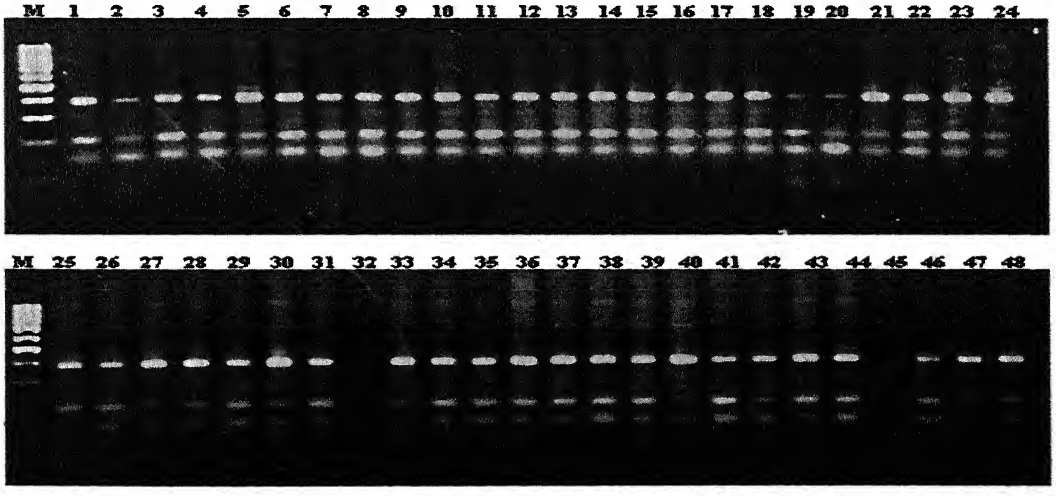

Fig. 21: SSR profile of 48 *ber* genotype with Z_aksdc3 (M: 100 bp ladder plus)

Z_aksdc4

The primer Z_akdc9 amplified 5 alleles with size of 93 bp, 97 bp, 313 bp, 338 bp and 618 bp. Four (80.0%) of these allele were polymorphic. The resolving power and PIC of the primer was 3.417 and 0.604, respectively. The primer was developed from the $(CT)_{14}$ repeat in the clone Zm01e04.y well.

Z_aksdc5

The primer Z_aksdc10 amplified 8 alleles with size of 126 bp, 162 bp, 279 bp, 344 bp, 507 bp, 659 bp, 746 bp and 829 bp. Five (62.5%) of these allele were polymorphic. The resolving power and PIC of the primer was 3.042 and 0.402, respectively. The primer was developed from the $(CT)_{14}$ repeat in the clone Zm01e04.z well.

Z_aksdc6

The primer Z_aksdc11 amplified 3 alleles with size of 59 bp, 81 bp and 187 bp. All of these alleles were monomorphic. The primer was developed from the $(AG)_7$ repeat in the clone Zm01g05.z well.

Z_aksdc8

The primer Z_aksdc17 amplified 6 alleles with size of 193 bp, 325 bp, 447 bp, 545 bp, 648 bp and 789 bp. Four (66.67%) of these allele were polymorphic. The resolving power and PIC of the primer was 2.125 and 0.39, respectively.

Z_aksdc9

The primer Z_aksdc18 amplified 2 alleles with size of 215 bp and 237 bp showing 100% polymorphism level (Fig 4.3.3). The resolving power and PIC of the primer was 1.417 and 0.769, respectively. The primer was developed from the $(TA)_7$ repeat in the clone Zm02c03.z well.

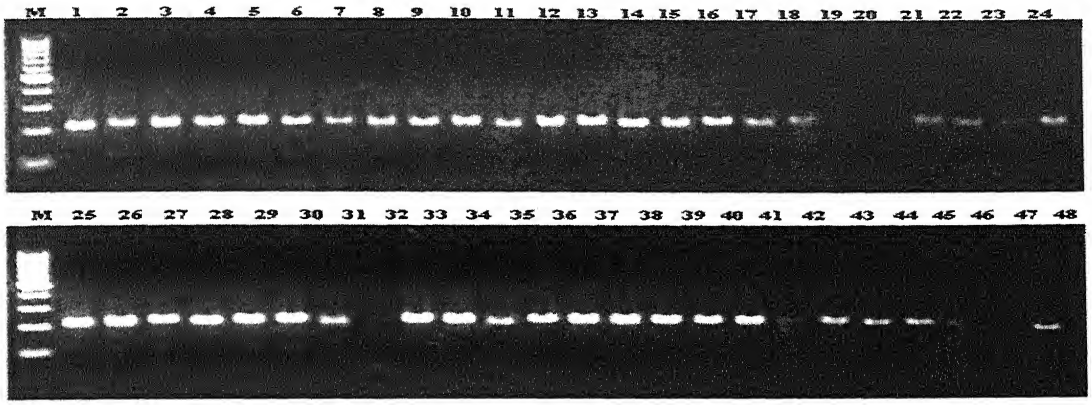

Fig. 22: SSR profile of 48 *ber* genotype with Z_aksdc9 (M: 100 bp ladder plus)

4.3.3 Genetic diversity based on microsatellite markers

The Jaccard's similarity values obtained between pairs of accessions ranged between 51.27% (between Pathan and Katha, Rohtak Safeda and Tikadi, Rohtak Safeda and Dandan, Nazuk and Thornless) and 100.00% (between Umraan and Kala Gola). A very high level of genetic similarity (96.42%) was observed between 5 diffrerent genotype pairs namely, Akhrota and Bawal sel.-2, Desi Alwar and Gola, Popular Gola and Rohtak Safeda, Kheera and Desi Alwar and Katha Rajasthan and Rohtak Safeda. Cluster analysis through UPGMA revealed 2 major clusters (Fig 22) - cluster I comprised of five genotypes, namely Katha, Tass-Batasso, Tikadi, Sanauri and Thornless, whereas cluster-II comprised of remaining 43 genotypes that were subgrouped into 3 major sublusters. In the cluster-II, subcluster II-b was smallest with 3 genotypes, namely Sanauri-3, Seb and No-A, whereas, the subcluster II-a and the subclsuter II-c contained 22 and 18 genotypes, respectively. The genotypes Gola, Popular Gola and Kala Gola, characteristically round in shape shared high genetic similarity between Gola and Popular Gola (86%), Popular Gola and Kala Gola (87%) and Gola and Kala Gola (76%). Based on morphological traits Katha, Katha Bombay, Katha Rajasthan and Umran have been reported to be different names of the same genotype in different regions. In the SSR based genetic diversity, these genotypes shared different level of genetic similarity as Katha and Katha Bombay (57%), Katha and Katha Rajasthan (55%), Katha and Umraan (67%), Katha Bombay and Katha Rajasthan (71%), Katha Bombay and Umraan (79%) and Katha Rajasthan and Umraan-82%. The genotypes Gola, Akhrota, Nazuk and Seb that were earlier reported to be different morphologically although with insignificant differences, shared

different levels of genetic similarity e.g. between Gola and Seb (81.0%), Gola and Nazuk (71%), Gola and Akhrota (74%), Seb and Nazuk (73%), Seb and Akhrota (52%), Nazuk and Akhrota (77%). The genotypes, Chuhara and Reshmi that were reported exactly similar based on their morphology shared 80% genetic similarity.

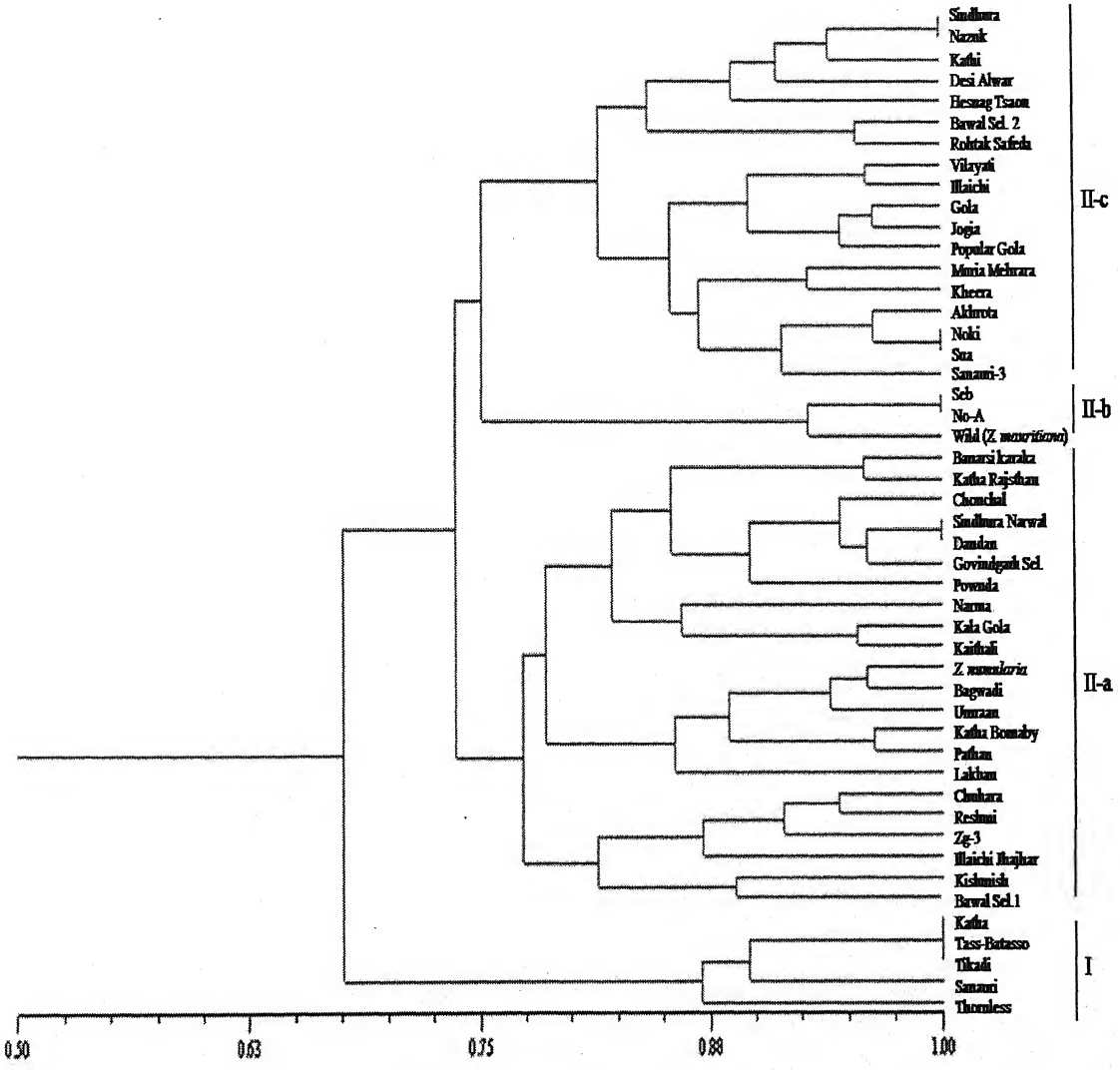

Fig. 23: Genetic relationship among 48 *ber* genotypes based on Jaccard's similarity coefficient for SSR analysis

4.4 Comparison of RAPD and ISSR Markers to Assess Genetic Diversity

The Mantel matrix correlation value (r) = 0.4196 (between RAPD and ISSR), 0.2221 (between RAPD and SSR) and 0.3168 (ISSR and SSR) suggest poor correlation between ISSR and RAPD marker system and very poor correlation of SSR with the RAPD and ISSR marker systems.

4.4.1 Relationship between Rp and PIC

The calculated Rp values for the three marker systems were plotted against PIC values and a linear relationship was observed between the two parameters (Fig. 23).

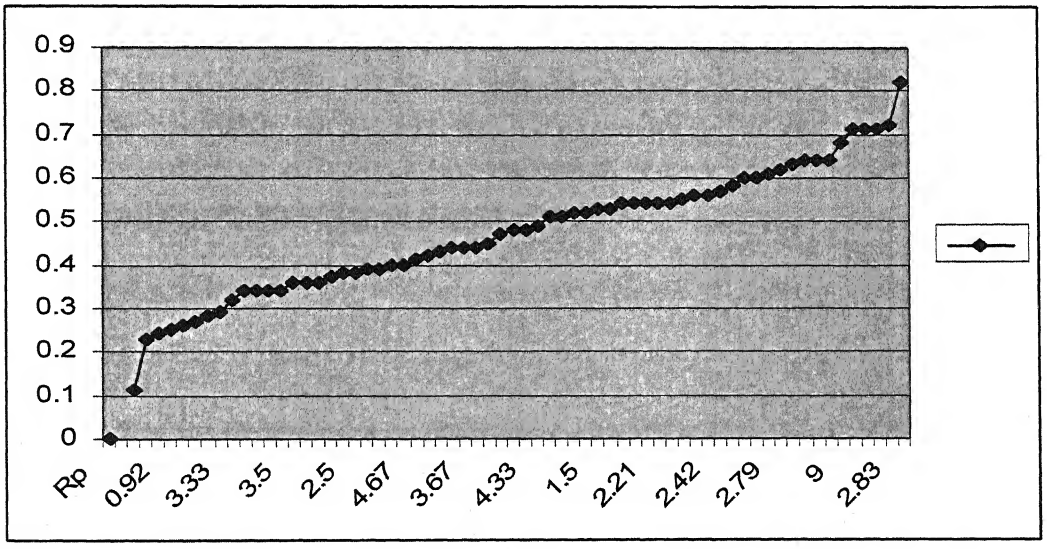

Fig. 24: Graphical relationship between Resolving Power (Rp) and Polymorphism Information Content (PIC)

4.5 Combined Profile of RAPD, ISSR and SSR Markers

The combined data for RAPD, ISSR and SSR was analysed for comprehensive grouping and establishing genetic relationships.

Cluster analysis through UPGMA revealed 4 major clusters (Fig. 24) - cluster I and cluster-II comprised of two genotypes each, namely, Tikadi, and Thornless, Seb and Chuahara, respectively. Cluster-III comprised of 4 genotypes Katha, Rohtak Safeda, Sindhura Narwal and Sanauri. The genotype, Katha, No-A and Sindhura were not grouped with other genotype, whereas, cluster-IV comprised of remaining 37 genotypes that were subgrouped into 4 major sublusters. In the subcluster-IVa seven genotypes Illaichi, Tass-Batasso, Umraan, Chonchal, Bagwadi, *Z. nummularia* and Sanauri-3, whereas, in subcluster IV-b with 5 genotypes, namely Pathan, Reshmi, Kishmish, Zg-3 and Illaichi Jhajhar, whereas, the subcluster IV-c contained 12 genotypes, namely Gola, dandan, Katha Bombay, akhrota, Jogia, Hesang-Tsaon, Kala Gola, Lakhan, Nazuk, Mundia Mehrara, Noki and Pownda, respectively. Fourteen genotypes namely, Vilayati, Banarasi Karaka, Narma, Desi Alwar, Kaithali, Govindgarh Sel., *Z. mauritiana*, Bawal Sel. 1, Bawal Sel. 2, Popular Gola, Sua, Katha Rajasthan and Kheera grouped in IV-d, whereas, the remaining two genotypes, No-A and Sindhura did not grouped with any of the genotypes.

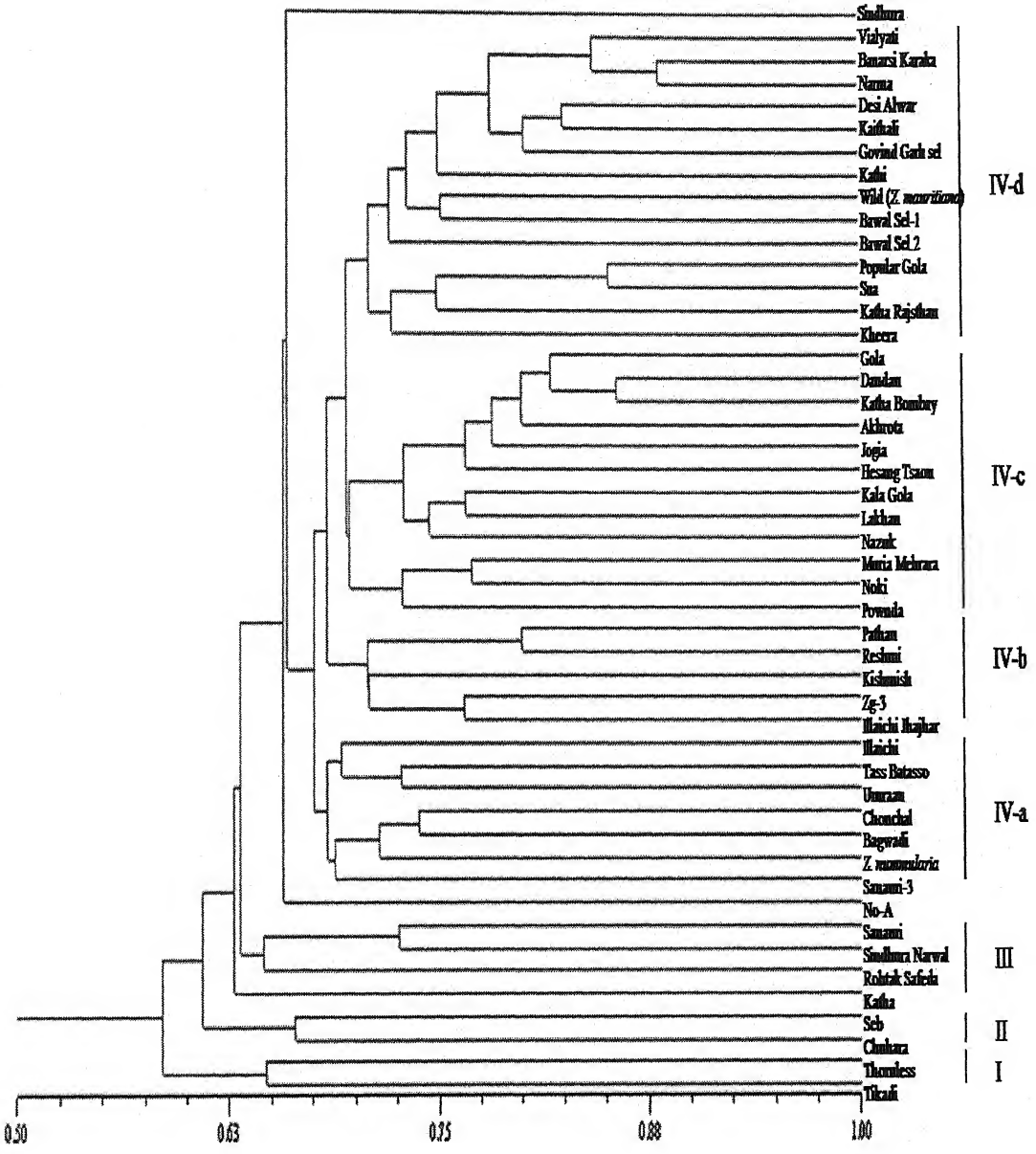

Fig. 25: Combined genetic relationship among 48 *ber* genotypes based on Jaccard's similarity coefficient

4.6 Development of DNA Fingerprinting Combining All the Marker (RAPD, ISSR and Microsatellite) Used

The genetic relationship established in the present study revealed distinguishing ability of RAPD primers to the instead phenotypically similar genotypes e.g. morphologically similar genotypes; Katha, Katha Bombay, Katha Rajasthan and Umaran were clustered into entirely different sub-clusters II-i, II-f, II-h and II-d, respectively and shared different level of genetic similarity. The results are in accordance to the suggestions made (Chadha *et al.* 1972). Moreover, all the primers except OPC-2, OPC-8, OPC-13, OPC-20, OPE-4 and OPF-7 amplified at least one different band to distinguish the four genotypes. ISSR primer-8, primer-10 and primer21 distinguished Katha, Katha Rajasthan and Umraan and the primer-23 distinguished Katha Bombay. All the ISSR primers were able to distinguish one or more of the four genotypes. The SSR primers The primer, Z_aksdc5 and Z_aksdc9 distinguished Katha and Z_aksdc10 and Z_aksdc17 distinguished Katha Bombay, whereas, the genotype Umraan and Katha Rajasthan could not be separated. The genotypes Gola, Akhrota, Seb and Nazuk earlier reported to be different morphologically although with insignificant differences grouped in different subclusters and were distinguishable with all the RAPD primers except OPC-4, OPC-13, OPC-16, OPE-4 and OPF-4. The ISSR primer-16, whereas, all other ISSR primers, except primer-6, primer-23 and primer-24 distinguished one or more of the four genotypes. The SSR primers Z_aksdc5 and Z_aksdc17 distinguished Seb and Z_aksdc9 distinguished Akhrota, whereas, the Z_aksdc10 distinguished Seb and Akhrota both. None of the primer distinguished Nazuk and Gola from other four genotypes. The genotypes Kala Gola, Popular Gola and Gola that are characteristically round in shape fell into different subclusters. Moreover, the earlier two genotypes were distinguished by 7 ISSR primers, viz.,

primer2, primer-3 primer-4, primer-5, primer-12, primer-23 and primer-26. The genotype, Gola was distinguished by primer-10, primer-11, primer-14, primer-21 and primer-22 with one of the two earlier genotypes. Except primer10, primer-21, primer-22 and primer-16 all ISSR primers were able to distinguish Kala gola. The ISSR primer-8 and primer-24 were unable to distinguish any of the four genotypes. Based on morphology, two genotypes Chhuhara and Reshmi are reported to be exactly same but in the present study Moreover, all the ISSR primers distinguished the two genotypes. The SSR primer Z_aksdc2 amplified a unique fragment of 324bp in Katha Bombay. Almost all the genotypes displayed different fingerprint with RAPD/ISSR/SSR or in combination.

Table 3: RAPD primers with sequences and the properties of amplified products in ber genotypes

Sl No.	Primer Designation	Sequence (5′ - 3′)	TNB	NPB	P%	Rp	PIC
1	OPC-4	5'-CCGCATCTAC-3'	6	4	66.67	3.00	0.44
2	OPC-3	5'-GGGGGTCTTT-3'	9	6	66.67	3.29	0.54
3	OPC-6	5'-GAACGGACTC-3'	8	7	87.50	3.67	0.51
4	OPC-7	5'-GTCCCGACGA-3'	7	5	71.43	2.33	0.40
5	OPC-13	5'-AAGCCTCGTC-3'	5	5	100.00	2.75	0.54
6	OPC-14	5'-TGCGTGCTTG-3'	6	6	100.00	1.50	0.53
7	OPC-2	5'-GTGAGGCGTC-3'	8	3	37.50	1.04	0.26
8	OPC-20	5'-ACTTCGCCAC-3'	6	4	66.67	1.83	0.48
9	OPC-8	5'-TGGACCGGTG-3'	7	4	57.14	3.08	0.47
10	OPC-9	5'-CTCACCGTCC-3'	3	3	100.00	0.58	0.39
11	OPC-11	5'-AAAGCTGCGG-3'	10	3	30.00	1.21	0.28
12	OPC-18	5'-TGAGTGGGTG-3'	10	6	60.00	3.50	0.36
13	OPC-16	5'-CACACTCCAG-3'	8	4	50.00	2.75	0.38
14	OPC-15	5'-GACGGATCAG-3'	11	9	81.82	5.92	0.71
15	OPC-10	5'-TGTCTGGGTG-3'	6	3	50.00	2.21	0.34
16	OPD-12	5'-CACCGTATCC-3'	6	6	100.00	1.96	0.60

17	OPF-1	5'-ACGGATCCTG-3'	11	6	54.55	4.67	0.41
18	OPD-11	5'-AGCGCCATTG-3'	12	10	83.33	5.42	0.52
19	OPD-3	5'-GTCGCCGTCA-3'	17	15	88.24	9.00	0.68
20	OPF-2	5'-GAGGATCCCT-3'	14	11	78.57	3.75	0.71
21	OPE-2	5'-GGTGCGGGAA-3'	10	7	70.00	3.29	0.54
22	OPF-11	5'-TTGGTACCCC-3'	12	9	75.00	3.83	0.61
23	OPD-8	5'-GTGTGCCCCA-3'	7	6	85.71	3.08	0.55
24	OPF-8	5'-GGGATATCGG-3'	11	11	100.00	5.75	0.71
25	OPF-7	5'-CCGATATCCC-3'	6	6	100.00	2.42	0.57
26	OPF-6	5'-GGGAATTCGG-3'	7	5	71.43	2.79	0.62
27	OPF-16	5'-GGAGTACTGG-3'	7	5	71.43	4.17	0.52
28	OPF-4	5'-GGTGATCAGG-3'	7	5	71.43	1.50	0.34
29	OP F-3	5'-CCTGATCACC-3'	9	7	77.78	4.04	0.48
30	OPE-14	5'-TGCGGCTGAG-3'	8	6	75.00	3.00	0.64
31	OPD-4	5'-TCTGGTGAGG-3'	10	9	90.00	4.54	0.64
32	OPE-11	5'-GAGTCTCAGG-3'	5	5	100.00	1.92	0.44
33	OPE-13	5'-CCCGATTCGG-3'	8	7	87.50	3.67	0.72
34	OPE-20	5'-AACGGTGACC-3'	8	8	100.00	3.75	0.45
35	OPE-19	5'-ACGGCGTATG-3'	8	6	75.00	4.33	0.49
36	OPE-4	5'-GTGACATGCC-3'	6	3	50.00	0.46	0.34
37	OPD-7	5'-TTGGCACGGG-3'	14	6	42.86	3.33	0.32
38	OPD-20	5'-ACCCGGTCAC-3'	7	2	28.57	0.92	0.11

39	OPE-6	5'-AAGACCCCTC-3'	9	8	88.89	5.54	0.58
40	OPE-9	5'-CTTCACCCGA-3'	4	4	100.00	2.83	0.82
41	OPF-15	5'-CCAGTACTCC-3'	10	9	90.00	3.96	0.63
42	OPF-13	5'-GGCTGCAGAA-3'	5	4	80.00	1.79	0.29
43	OPF-12	5'-ACGGTACCAG-3'	9	6	66.67	2.21	0.54
44	OPF-10	5'-GGAAGCTTGG-3'	6	4	66.67	2.13	0.56
45	OPE-7	5'-AGATGCAGCC-3'	3	2	66.67	0.92	0.25
46	OPF-9	5'-CCAAGCTTCC-3'	2	1	50.00	0.96	0.39
	Total		**368**	**271**	-	-	-
	Average		**8**	**5.89**	**74.15**	**3.06**	**0.49**

*TNB= Total number of bands; NPB= Number of polymorphic bands; P%= Polymorphism percentage; Rp= Resolving power; PIC= Polymorphic information content

Table 4: ISSR primers with sequences and the properties of amplified products in *ber* genotypes

S No.	UBC[1] Primer	Sequence (5/ - 3/)	TNB	NPB	P%	Rp	PIC
1	808	AGAGAGAGAGAGAGAGC	11	9	81.8	4.042	0.34
2	809	AGAGAGAGAGAGAGAGG	11	10	90.9	4.75	0.37
3	814	CTCTCTCTCTCTCTCTA	10	10	100	4.54	0.42
4	825	ACACACACACACACACT	9	6	66.6	2.34	0.23
5	829	TGTGTGTGTGTGTGTGC	12	10	83.3	4.21	0.51
6	840	GAGAGAGAGAGAGAGACTT	10	10	100	2.5	0.4
7	841	GAGAGAGAGAGAGAGACTC	10	10	100	5.29	0.53
8	848	CACACACACACACACAAGG	7	7	100	3.67	0.44
9	850	GTGTGTGTGTGTGTGTCTC	8	8	100	4.71	0.64
10	854	TCTCTCTCTCTCTCTCAGG	9	9	100	2.5	0.38
11	855	ACACACACACACACACCTT	10	9	90.0	3.79	0.6
12	856	ACACACACACACACACCTA	4	4	100	3.67	0.36
13	876	GATAGATAGACAGACA	9	9	100	3.71	0.36
14	880	GGAGAGGAGAGGAGA	9	7	77.8	5.63	0.54
15	889	AGTCGTAGTACACACACACACAC	7	4	57.1	2.04	0.24

16	890	ACGACTACGGTGTGTGTTTGTGT	9	9	100	1.00	0.56
17	894	TGGTAGCTCTTGTCAGGCAC	12	11	91.6	5.17	0.43
18	900	ACTTCCCCACAGGTTAACACA	10	8	80.0	2.63	0.27
		Total	**167**	**152**	-	-	
		Average	**9.28**	**8.44**	**89.94**	**3.68**	**0.42**

*TNB= Total number of bands; NPB= Number of polymorphic bands; P%= Polymorphism percentage; Rp= Resolving power; PIC= Polymorphic information content ([1]- University of British, Columbia)

Table 5: Novel SSR primers with sequences and the properties of amplified products in *ber* genotypes

Primer Designation	Primer sequence (5′-3′) Forward	Reverse	RM	TNB	NPB	P%	PIC	Rp
Z_aksdc1	GGGTGAAATAAAGGAGAGATTAATTG	CCATCTTTCTACTCTATCCACCA	$(TG)_5$	1	0	0.00	0	0
Z_aksdc2	AAAACCATGATAAGGCCAAAAGT	AACCAGCAATGGTTTTTGAC	$(GA)_5$	2	1	50.00	0.50	0.42
Z_aksdc5	TAGTGAATTCTCTTGCTTAGATCTGGA	GGTTAAAGAGAGAAACTTGCATACG	$(TA)_5$	5	1	20.00	0.09	0.54
Z_aksdc9	CGCAAAAAGAAAGACATAATGAA	GAGAGAGTGACAGCGGGTTT	$(CT)_{14}$	5	4	80.00	0.60	3.42
Z_aksdc10	GAGAGAGTGACAGCGGGTTT	ATAGGTGTGGGTGTGGGAGA		8	5	62.50	0.40	3.04
Z_aksdc11	CACATGGCCTAGACATCTCTGAT	TCGCTTCGAAGTTTCTGTCA	$(AG)_7$	3	0	0.00	0	0
Z_aksdc16	GGGGCGATCCTAGTGATTCT	CCGGCTCTCTTGTGTTTC		**Not amplified**				
Z_aksdc17	ATCAGCAATCATTGGCATCAA	TGGATTTTGTGGTGGCTGTA		6	4	66.67	0.39	2.13
Z_aksdc18	TGCACATTTGCTCATGTCTC	TGAAGGTTGATGGCATGTGA	$(TA)_7$	2	2	100.00	0.77	1.42
Z_aksdc21	AGAGCTGTACCAGTGAAAACCA	GGTAGATTCGCATCCCGTTT	$(GT)_5$	**Not amplified**				
	Total			**28**	**17**	**-**	**-**	**-**
	Average			**-**	**-**	**63.19**	**0.46**	**1.83**

*RM= repeat motif; TNB= Total number of bands; NPB= Number of polymorphic bands; P%= Polymorphism percentage; Rp= Resolving power; PIC= Polymorphic information content

Chapter 5

DISCUSSION

Ziziphus species are distributed over wide geographical and climatic condition that is indicative of its tremendous genetic diversity, which needs to be identified and catalogued. Molecular markers based on polymerase chain reaction (PCR) method offer several advantages over the sole use of conventional morphological markers. The PCR technology has led to the development of two simple and quick techniques *viz.,* random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR). Both the techniques are inexpensive and readily adaptable for routine germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes. The microsatellite/ SSR markers are now being marker of choice because of its highly polymorphic, reproducible, co-dominant nature and distribution throughout the genome (Sharma, 2005). SSR markers have also been used for varietal identification (Smith, 1994), genetic fingerprinting (Luro *et al.*, 2000) and for construction of genetic linkage maps (Sharma, 2005).

Initially, the optimization for DNA isolation and PCR components was done because there was no report of molecular work on *ber*. It was necessary to validate and to standardize the protocols for DNA isolation/ PCR analysis of *ber* that has been described as one of the 'Fruits for the future' (Pareek, 2001). For the DNA isolation minor modification to the protocol described by Doyle and Doyle (1990) were applied as higher CTAB concentration (5%) was used. This higher concentration of CTAB was observed to provide high concentration of DNA. This has been possibly due to complete cell-wall disruption to exude out the genomic DNA from hard tissues. Since there are no reports on *ber* molecular studies except the recent publications by our group (Devanshi *et al.*, 2007,

Singh *et al.*, 2006 and 2007) on *Z. mauritiana* and *Z. nummularia* and Weekly *et al* (2002) on *Z. celata.* The results obtained from the present study will be discussed with reference to other crops preferably to fruit/woody crops under the following subhead:

5.1 RAPD Analysis

For the RAPD analysis in the present study PCR conditions were optimized. The $MgCl_2$ (2.0 mM) in the RAPD-PCR reaction was optimized. The results are in agreement to Wolf *et al.* (2001) based on their study on grape. The concentration of $MgCl_2$ have been optimized for RAPD-PCR reaction in many crops (Sant *et al.*, 1999 and Singh *et al.*, 2007) and been reported to provide the best results with 1.5 mM (Sant *et al.*, 1999 and Birmeta *et al.*, 2004) to 3.5 mM (Offel *et al.*, 2004). Similarly, 1 Unit of Taq DNA polymerase have been observed to provide the scorable and reproducible bands, whereas, in other crops 0.5U (Virk *et al.*, 1995) in rice to 4U (Wolf *et al.*, 2001) in grapevine have been reported as optimum. The 200 µM of dNTPs, optimized for RAPD-PCR in *ber* is supported by Zhuang *et al.* (2004) in *Cucumis sp.*, Wolf *et al.* (2001) in grapevine and Singh *et al.* (2007) in *ber*, whereas, in apple 100 µM have been reported the optimum (Koller *et al.*, 1993). For the selection of polymorphic primers with minimal inputs, 4 bulks of DNA were used. Use of bulks for selection of polymorphic primers in gene mapping studies have been reported (Sharma, 2005).

The 46 RAPD primers amplified 368 bands, of these 271 (74.15%) were polymorphic. This level of polymorphism through RAPD has been reported by Vidal *et al.* (1999) in grapevine (69.2%) and Zhang *et al.* (2005) in *Nicotiana* (63.1%). In contrast, a higher level of polymorphism in cotton and *Cocoyam* (97.21% and 93.33%, respectively) have

been reported (Rana and Bhatt, 2005 and Offel *et al.*, 2004) and a very low level of polymorphism (9.3%) have been reported by Yang and Quiros (1993) in *Apium*. The level of polymorphism depends on the primers used, number of bands amplified and the material analysed for the diversity. The minimal number of RAPD bands has been suggested as 200 to obtain accurate estimates of genetic similarity (Guirao *et al.*, 1995). The resolving power (Rp) (number of genotypes resolved by individual primer) for the RAPD primers in the present study is an average of 3.06 per primer. We have reported the similar level of Rp values for the RAPD primer (Devanshi *et al.*, 2007). The Rp of a primer has been reported to be correlated with its ability to differentiate genotypes (Prevost and Wilkinson, 1999). The primers with high Rp values OPC-15, OPF-8, OPE-6 and OPD-11 (5.92, 5.75, 5.45 and 5.42, respectively) were able to distinguish more number of *ber* genotypes. The polymorphism information content (PIC) values have been reported to be a suitable index of identifying superior primers for genetic diversity and relationship studies. In this study, polymorphism information content (PIC) ranged from 0.11 (OPD-20) to 0.82 (OPE-9) with an average of value of 0.49 per primer. The average PIC value per primer agrees with the earlier report (0.55) on *Avena sp.* (Li *et al.*, 2001) and with a range between 0.09 to 0.67 in tomato (He *et al.*, 2003b).

RAPD markers have proved its ability to differentiate and to fingerprint different genotypes that are phenotypically similar. The RAPD analysis revealed high level of genetic similarity ranging from 26.3% to 78.9% among the *ber* genotypes studied. In the literature low range of genetic diversity, 70% to 90% (genetic similarity) has detected by RAPD have been reported in papaya (Stiles *et al.*, 1993) and a very high range, 27% to

93% have been reported in strawberry (Degani *et al.*, 2001). The level of genetic similarity is not an index for marker system but it mostly depends on the material used for diversity.

5.2 ISSR Analysis

For the ISSR analysis dNTP, Taq DNA polymerase and $MgCl_2$ at concentrations of 200 µM, 1 unit and 1.5 mM respectively provided good amplification. The results for dNTPs and $MgCl_2$ are in accordance to Herrera *et al.*, (2002) in grapes and Huang and Sun (2000) in *Ipomea*. However, the Taq polymerase has been reported to provide good amplification even at lower concentration of 0.75U (Singh *et al.*, 2007b) in bittergourd. For the selection of polymorphic primers with minimal inputs, 4 bulks of DNA were used. Use of bulks for selection of polymorphic primers in gene mapping studies have been reported (Sharma, 2005).

The 18 ISSR primers amplified, 167 fragments, of these 152 (89.94%) were polymorphic. In Grapes, The level of polymorphism through ISSR analysis has been reported by Singh *et al.* (unpubl.) in grapes 67.7%. The level of polymorphism depends on the primers used, number of bands amplified and the material analysed for the diversity.

The resolving power (Rp) (number of genotypes resolved by individual primer) for the ISSR primers in the present study is an average of 3.68 per primer. We have reported the similar level of Rp values for the ISSR primer (Devanshi *et al.*, 2007). The Rp of a primer has been reported to be correlated with its ability to differentiate genotypes (Prevost and Wilkinson, 1999). The primers with high Rp values UBC-841, UBC-894, UBC-809 and UBC-850 posses high Rp values of 5.29, 5.17, 4.75 and 4.71, respectively

were able to distinguish more number of *ber* genotypes. The polymorphism information content (PIC) values have been reported to be a suitable index of identifying superior primers for genetic diversity and relationship studies Polymorphism information content (PIC) ranged from 0.23 (UBC-825) to 0.64 (UBC-850) with an average of value of 0.42 per primer.

5.3 Development of SSR Markers Using Enriched Genomic Library

The genomic sequences were enriched through 5′-biotinylated repeats $[GA]_{20}$, $[CA]_{20}$, $[AGA]_{15}$, $[ACA]_{15}$, $[CAT]_{15}$ and $[GATA]_{20}$ repeats. The frequencies of di-, tri-, tetra- and pentamer repeats were calculated considering sequence complementarity. Out of the repeat motifs identified at ≥ 5 repeats, the dimer repeats were highest (7), followed by trimer and tetramer (1) repeats. The results are in agreement of Kolliker *et al.* (2001) for higher number of dimer repeats. For the dimer repeats identified in the enriched sequences, the repeat $(CT/AG)_n$, $(CA/TG)_n$, $(TA)_n$ and $(GA/TC)_n$ were enriched in the sequenced clone as compared to other dimer repeats. Similar enrichment of dimer repeats of $(CT)_n$ in peach (Aranzana *et al.*, 2002) and Citrus (Ahmad *et al.*, 2003) have been reported. The repeat $(CA)_n$ found enriched through enriched library method in *Avena* sp. (Li *et al.*, 2001), *Trifolium repens* (Kolliker *et al.* 2001) and Citrus (Ahmad *et al.*, 2003), whereas, $(GA)_n$ enrichment in *Avena sp* (Li *et al.* 2001) and in peanut (He *et al.*, 2003a). The dimer repeats, $(AC)_n$ and $(GA)_n$ have been reported abundant in plant species by Gupta *et al.* (1996). In the trimer repeats, $(AGA)_n$ was enriched most, followed by other trimers including $(ACA)_n$. The trimer $(ACA)_n$ was enriched in the genomic DNA library constructed in the present study. The enrichment of trimer repeats $(CTT)_n$ have been

reported by Ahmad *et al.* (2003) from the library enriched for the $(CTT)_n$ repeat. Among tetramer repeat, the genomic library were enriched for $(GATA)_n$ sequence, but in the enriched sequenced clones, the repeat $(AATT)_n$ and $(CGGC)_n$ was most abundant followed by $(ATGA)_n$, $(ATAA)_n$, $(CCAA)_n$, $(CTTC)_n$, $(TAAA)_n$ and $(TTCA)_n$ Although no pentamer repeat was enriched using biotinylated columns. However, the pentamer repeat $(CCAAG)_n$ was observed (7.14%) of the total pentamers, the most possible reason of identification of high number of pentamer repeat being the selection criteria (very low number of repeat motif = 2), whereas, it was higher for other repeat motifs. Moreover, increasing the stringency of the repeat selection criteria on SSRIT (2 to 3), no pentamer was observed.

Of the 10 primers developed, 71.4% primers were from the repetitive elements (5 out of 7) and 33.3% (1 of three) from non-repetitive elements were polymorphic. The results contrast to earlier reports on peanut with 33.9% polymorphism (He *et al.*, 2003a) for microsatellite regions and *Avena sp.* (Li *et al.*, 2001) as that reported only 25% of primers from repetitive elements and 46% of the non-repetitive elements showed polymorphism.

5.4 Genetic Fingerprinting Based on the Three Marker Systems

RAPD, ISSR and SSR markers have proved their ability to differentiate and to fingerprint different genotypes that are phenotypically similar and revealed high level of genetic diversity among the *ber* genotypes studied. In the literature, RAPD has been reported to show low range of genetic similarity, 70% to 95% in papaya (Stiles *et al.*, 1993) and ISSR as 62.2% in Ipomea (Hunag and Sun, 2000) and a very high range of genetic diversity through RAPD have been reported in Indian sapota (Meghala *et al.*, 2005). The

level of genetic similarity depends on the material used. The distinguishing ability of RAPD and ISSR markers for the morphologically similar genotypes; Katha, Katha Bombay, Katha Rajasthan and Umaran has been established as these genotypes clustered into entirely different sub-clusters and shared different level of genetic similarity. The results are in accordance to the suggestions made (Chadha *et al.* 1972). Moreover, all the RAPD primers except OPC-2, OPC-8, OPC-13, OPC-20, OPE-4 and OPF-7 amplified at least one different fragment to distinguish the four genotypes. ISSR primers, UBC829, UBC841 and UBC890 distinguished Katha, Katha Rajasthan and Umraan and the UBC880 distinguished Katha Bombay. All the ISSR primers were able to distinguish one or more of the four genotypes. Z_aksdc5 and Z_aksdc9 distinguished Katha and Z_aksdc10 and Z_aksdc17 distinguished Katha Bombay, whereas, the genotype Umraan and Katha Rajasthan could not be separated. Similarly, the other phenotypically similar genotypes Gola, Akhrota, Seb and Nazuk, Reshmi and Chuhara could be distinguished with RAPD, ISSR and SSR markers. These results suggest that standard set of primers combining RAPD and/or ISSR and/or SSR should be established to distinguish and characterize most of the *ber* genotypes. The similar suggestions have been made for RAPD markers by Koller *et al.* (1993). In addition, specific fragments were amplified in some genotypes with the three marker systems e.g. Thornless by OPD-4 and Tikadi by OPE-11 and OPE-13

In the present study, the *Z. nummularia* has been grouped with *Z. mauritiana.* In our earlier study on AFLP analysis of *ber* (Singh *et al.*, 2006), we have reported grouping of all the wild accessions of *Z. nummularia* together into a different sub-cluster away from

Z. mauritiana. This is because AFLP primers amplified more number of loci (952 loci) than the present RAPD (368 bands), ISSR (167 loci) and SSR (32) analyses. The overall study with RAPD, ISSR and SSR marker systems has revealed a broad genetic base in *Ziziphus species*. One of the major contributing factors to the high degree of genetic diversity observed in *ber* may be due to its evolutionary status as an outcrossing species. Similar observations have been made in Coconut (Perera *et al.*, 1998), neem (Singh *et al.*, 1999) and *ber* (Singh *et al.*, 2006) where the level of genetic diversity was shown to correlate with the breeding nature of the plants. In the study, the groupings of the cultivars did not, however, correspond to their eco-geographical regions. For instance, cultivars from Rajsthan, Punjab, Uttar Pradesh and Haryana remained together. This may be explained by the fact that human interventions have played a major role in varietal distribution of *ber* followed by cross-pollination between local and introduced materials. The groupings of samples in one cluster collected from different sub-zones have been reported in neem by Ranade *et al.* (2002) and Deshwal *et al.* (2005). Our study revealed that the phenotypically similar genotypes could be reliably distinguished genetically using the three marker system.

5.5 Comparison of RAPD, ISSR and SSR Marker Systems

The Mantel matrix correlation value (r) = 0.42 (between RAPD and ISSR), 0.22 (between RAPD and SSR) and 0.31 (ISSR and SSR) suggest poor correlation between ISSR and RAPD marker system and very poor correlation of SSR with the RAPD and ISSR marker systems. Negative correlations have been reported between RAPD and ISSR in Indian cashew (Archak *et al.*, 2003). A very high level of correlation between RAPD and SSR

markers have been reported by Ritschel *et al.* (2004) in melon and Zhuang *et al.* (2004) in *Cucumis sp.*. The comparison between the three marker systems suggest that the ISSR markers are more efficient than RAPD because of their capacity to reveal several informative bands in a single amplification (a mean of 8.44 informative bands per primers) as compared to 5.89 for RAPD and higher Rp (3.68 vs. 3.06) than RAPD primers (Fig. 3). Similar results have bee reported in barley using 10 ISSR and 10 RAPD primers (Fernandez *et al.*, 2002). Herrera *et al.* (2002) have also reported higher Rp value of the ISSR markers as compared to RAPD markers. Comparison of SSR markers to the RAPD and ISSR marker system suggested superiority of RAPD and ISSR over SSR markers due to lower average Rp and PIC values for SSR being 1.83 and 0.46, respectively. The results are in contrast to most of the reports on comparison of the three markers system (He *et al.*, 2003a). Possible reason for the result being the less number of bands detected by SSR markers (32) in comparison to RAPD (368) and ISSR (167). Moreover, RAPD and ISSR being arbitrary in nature and utilizing low annealing temperature, amplify spurious fragments, whereas, SSR being sequence dependent reflects true picture of the genome but requires higher number of primers for complete coverage of the genome. Contrast to the result, Luro *et al.* (2000) have reported very high efficiency of SSR markers (8 primers) to distinguish different groups of citrus, whereas, the RAPD and SSR markers have been reported equally capable to differentiate between watermelon genotypes (Jarret *et al.*, 1997). Although SSR amplify less average fragments as compared to RAPD/ISSR in the present study, but it was more reproducible as also reported by He *et al.* (2003a) in peanut and a single primer pair itself could distinguish

one or more genotypes e.g. Z-aksdc10 distinguished Seb and Akhrota both and Z_aksdc2 distinguished genotype Katha Bombay.

The Rp values and PIC values showed linear relationship. The results suggest both the parameters specify the superiority of one primer over other and there is no need to calculate both. The results are in agreement of Huang *et al.* (2002), whereas, contrast to Prasad *et al.* (2000) based on their study on wheat.

5.6 Future Implication of the Present Study

The present study opens a new vista for *ber* breeders and the taxonomists to review and resolve the mis-nomination of *ber* genotypes based not only morphologically but also using molecular markers. The markers (RAPD, ISSR and SSR) identified in this study for specific genotypes have immediate practical application for development of superior genotypes using diverse parents through molecular markers. This study would help the consideration of molecular fingerprinting using genotype specific fragments for germplasm conservation, rectification, purification and the identification of genotypes as this crop has been assigned as 'Future Crop' by Food and Agricultural Organization.

Chapter 6

SUMMARY

In the present study, genetic relationship and fingerprinting of 48 *ber* genotypes was done using three marker systems- random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and SSR markers. Since there is no established SSR marker system for *ber*, this study also aimed to develop novel SSR markers for *ber* through SSR-enriched library. The developed markers were used for polymorphic survey and genetic relationship study.

Salient findings are summarized as below:

(A) RAPD analysis

1. The 46 RAPD primers amplified 368 bands, of these 271 (74.15%) were polymorphic.
2. The resolving power (Rp) (number of genotypes resolved by individual primer) for the RAPD primers in the present study is an average of 3.06 per primer. The primers with high Rp values OPC-15, OPF-8, OPE-6 and OPD-11 (5.92, 5.75, 5.45 and 5.42, respectively) were able to distinguish more number of *ber* genotypes.
3. The polymorphism information content (PIC) ranged from 0.11 (OPD-20) to 0.82 (OPE-9) with an average of value of 0.49 per primer.
4. The RAPD analysis revealed high level of genetic similarity ranging from 26.3% to 78.9% among the *ber* genotypes studied.

(B) ISSR analysis

1. The 18 ISSR primers amplified, 167 fragments, of these 152 (89.94%) were polymorphic.

2. The resolving power (Rp) (number of genotypes resolved by individual primer) for the ISSR primers in the present study is an average of 3.68 per primer. The primers with high Rp values UBC-841, UBC-894, UBC-809 and UBC-850 posses high Rp values of 5.29, 5.17, 4.75 and 4.71, respectively were able to distinguish more number of *ber* genotypes.
3. The polymorphism information content (PIC) ranged from 0.23 (UBC-825) to 0.64 (UBC-850) with an average of value of 0.42 per primer.
4. The ISSR analysis revealed high level of genetic similarity ranging from 43.1% to 90.3% among the *ber* genotypes studied.

(C) Development of SSR markers using enriched genomic library

1. The genomic sequences enriched through 5′-biotinylated repeats $[GA]_{20}$, $[CA]_{20}$, $[AGA]_{15}$, $[ACA]_{15}$, $[CAT]_{15}$ and $[GATA]_{20}$ repeats. Out of the repeat motifs identified at ≥ 5 repeats, the dimer repeats were highest (7), followed by trimer and tetramer (1) repeats.
2. For the dimer repeats identified in the enriched sequences, the repeat $(CT/AG)_n$, $(CA/TG)_n$, $(TA)_n$ and $(GA/TC)_n$ were enriched in the sequenced clone as compared to other dimer repeats.
3. In the trimer repeats, $(AGA)_n$ was enriched most, followed by other trimers including $(ACA)_n$. The trimer $(ACA)_n$ was enriched in the genomic DNA library constructed in the present study.
4. The tetramer repeat $(AATT)_n$ and $(CGGC)_n$ was most abundant followed by $(ATGA)_n$, $(ATAA)_n$, $(CCAA)_n$, $(CTTC)_n$, $(TAAA)_n$ and $(TTCA)_n$.

5. The pentamer repeat $(CCAAG)_n$ was observed (7.14%) of the total pentamers.
6. Of the 10 pairs of SSR primers developed, 71.4% primers were from the repetitive elements (5 out of 7) and 33.3% (1 of three) from non-repetitive elements were polymorphic.

(D) SSR analysis

1. Of the 10 primers developed, 6 (60%) were polymorphic.
2. The resolving power (Rp) (number of genotypes resolved by individual primer) for the SSR primers in the present study is an average of 1.83 per primer. The primers with high Rp values Z_aksdc9, Z_aksdc10 and Z_aksdc17 posses high Rp values of 3.42, 3.04 and 2.13, respectively.
3. The polymorphism information content (PIC) ranged from 0.09 (Z_aksdc5) to 0.77 (Z_aksdc18) with an average of value of 0.46 per primer.
4. The SSR analysis revealed high level of genetic similarity ranging from 51.3% to 100.00% among the *ber* genotypes studied.

(E) Genetic fingerprinting based on the three marker systems

1. All the RAPD primers except OPC-2, OPC-8, OPC-13, OPC-20, OPE-4 and OPF-7 amplified at least one different fragment to distinguish the four genotypes.
2. ISSR primers; UBC829, UBC841 and UBC890_distinguished Katha, Katha Rajasthan and Umraan and the UBC880 distinguished Katha Bombay. All the ISSR primers were able to distinguish one or more of the four genotypes.

3. Z_aksdc5 and Z_aksdc9 distinguished Katha and Z_aksdc10 and Z_aksdc17 distinguished Katha Bombay
4. The sets of primers combining RAPD and/or ISSR and/or SSR should be established to distinguish and characterize most of the *ber* genotypes.
5. In the study, *Z. nummularia* grouped with *Z. mauritiana*.
6. The groupings of the cultivars did not correspond to their eco-geographical regions. For instance, cultivars from Rajsthan, Punjab, Uttar Pradesh and Haryana remained together.
7. Comparison of SSR markers to the RAPD and ISSR marker system suggested superiority of RAPD and ISSR over SSR markers due to lower average Rp and PIC values for SSR being 1.83 and 0.46, respectively.
8. The Rp values and PIC values showed linear relationship.

The present study suggests *ber* breeders and the taxonomists to review and resolve the mis-nomination of *ber* genotypes using molecular markers also. This study would help the consideration of molecular fingerprinting using genotype specific fragments for germplasm conservation, rectification, purification and the identification of genotypes of *ber* that has been assigned as 'Future Crop' by Food and Agricultural Organization.

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APPENDIX

Appendix-I

Composition of chemical reagents / solutions

Plant DNA isolation, purification and quantification:
DNA isolation and purification was carried out using CTAB (Cetyltrimethyl ammonium bromide) method, (Murray and Thompson, 1980)

Reagents:

(i) NaCl (5 M) (1 L)
292.2 g NaCl was dissolved in 800 ml prewarmed double distilled water, volume was adjusted up to 1 L with double distilled water and solution was autoclaved.

(ii) Tris-Cl (1 M) pH 8.0 (1 L)
121g Tris-Cl salt was dissolved in 800 ml double distilled H_2O and pH was adjusted using concentrated HCl to 8.0. The volume was adjusted up to 1000 ml and solution was autoclaved.

(iii) CTAB (10%) (1 L)
100.0 g of CTAB was dissolved in 800 ml of double distilled water, volume adjusted up to 1000 ml and solution was autoclaved.

(iv) EDTA-Na_2 (0.5 M) pH 8.0 (100 ml)
18.61 g sodium salt of EDTA was dissolved in 80 ml double distilled H_2O and pH was adjusted using NaOH pellets to 8.0. The volume was brought up to 100 ml and solution was autoclaved.

DNA extraction buffer: (100 ml)

1 M Tris-Cl(pH 8.0)	10.0 ml	(100 mM)	
0.5 M EDTA (pH 8.0)	4.0 ml	(20 mM)	
5 M NaCl	28 ml	(1.4 M)	
CTAB (10%)	20 ml	(2%)	
β-Mercaptoethanol	200 µl	(0.2%)	- added fresh

(v) Ethanol (70%) (100 ml)

(vi) Chloroform: isoamyl alcohol (24:1)

(vii) Sodium acetate (3 M) pH 5.2 (100 ml)
2000 g of sodium acetate

(vii) Tris-EDTA buffer10:1(TE) pH 8.0 (100 ml)
Tris(10mM):EDTA(1mM) buffer was prepared in 100 ml as follows:
1mM Tris-Cl, pH 8.0 1 ml

0.5M EDTA pH 8.0 0.1 ml
Autoclaved double distilled H_2O 98.8 ml

(viii) 50X TAE pH 8.0 (100 ml)
242 g of tris base was dissolved in 400 ml water , 57.1 ml Glacial acetic acid and 0.5 M EDTA and pH was adjusted to 8.5. The volume was brought up to 1000ml and solution was autoclaved.

(ix) 10X TBE pH 8.0 (400 ml)

(x) Ethidum bromide (10 mg/ ml)

(xi) DNA loading dye pH 8.0 (10 ml)
Bromophenol blue 0.12 g (0.25%)
Xylene cyanol 0.12 g (0.25%)
30% glycerol in water
store at 4^0C.

(xii) RNase (10 mg/ml)
10 mg RNase A was dissolved in 1ml autoclaved double distilled H_2O, vortex well and boil in water bath at 100°C for 10-15 minutes, cool down and store at -20°C.

(xiv) Phenol: chloroform: isoamyl alcohol (25:24: 1) v/v.

Appendix- II

Chemicals /equipments used and their sources

I. Reagents and Chemicals

Item	Source
Chemicals:	
Tris-Cl	Sigma
NaCl	Sigma
EDTA-Na_2	Sigma
CTAB	USB
β Mercaptoethanol	Sigma
PVP	Qualigens
Isopropanol	Qualigens
Chloroform: Isoamyl alcohol (24:1) mixture	E- Merck
Ethanol	E- Merck
Taq DNA polymerase	MBI-Fermentas
10-X PCR buffer	MBI-Fermentas
$MgCl_2$	MBI-Fermentas
dNTP mix	MBI-Fermentas
STMS primers	Life Technologies, GCC
λ- DNA	MBI-Fermentas
DNA ladder (Gene ruler 100 bp)	MBI-Fermentas
Agarose (molecular biology grade)	Sigma
Ethidium bromide solution	Sigma
Loading dye (6X)	Sigma

Instruments:

Microfuge	Plasto Grafts
Centrifuge	Sorvall RC-5C
Micropippets	Nichiryo/Gilson/Eppendorf
Waterbath	Onbitek
Deep freezer (-20°C)	Samsung
Refrigerator	Kelvinator
Microwave-oven	LG

contd...

Dy NA Quant 200 flourimeter	Hoefer
Quarts cuvette	Sigma
Eppendorf tubes	Quiagen
pH meter	Systronics
Vortex	Tarsons
Water purification system	Millipore
Thinwalled PCR tubes	Tarson
PCR machine	Eppendorf/Biometra/ ABI
Laminar flow	Atlantis
Gel electrophoresis unit	Alpha Tech
Power Pack 300	Biorad
Lab Shaker	Kuhner

Appendix III

Sequences from 21 repeats and 6 primers (175 readable sequences, 78 sequences >50% readable,23 sequence >90%)

>Zm01a01.y well A01 Zm1-2.y.z Run01 Cimarron 3.12 225 (324/1277)---25.37%
>Zm01a01.z well A13 Zm1-2.y.z Run01 Cimarron 3.12 123 (123/1461)--- 8.42%
>Zm01a02.z well B13 Zm1-2.y.z Run01 Cimarron 3.12 18
>Zm01a03.y well A02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01a03.z well A14 Zm1-2.y.z Run01 Cimarron 3.12 586 (586/618)--- 94.82%
>Zm01a04.y well B02 Zm1-2.y.z Run01 Cimarron 3.12 651 (651/894)--- 72.82%
>Zm01a05.z well A15 Zm1-2.y.z Run01 Cimarron 3.12 846 (846/848)--- 99.76%
>Zm01a06.y well B03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01a06.z well B15 Zm1-2.y.z Run01 Cimarron 3.12 651 (669/749)--- 89.31%
>Zm01a07.y well A04 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01a07.z well A16 Zm1-2.y.z Run01 Cimarron 3.12 496 (496/2519)--- 19.69%
>Zm01a08.y well B04 Zm1-2.y.z Run01 Cimarron 3.12 701 (701/1100) --- 63.72%
>Zm01a08.z well B16 Zm1-2.y.z Run01 Cimarron 3.12 564 (570/583)--- 97.77%
>Zm01a09.y well A05 Zm1-2.y.z Run01 Cimarron 3.12 381 (381/964)--- 39.52%
>Zm01a09.z well A17 Zm1-2.y.z Run01 Cimarron 3.12 160 (287/647)--- 44.36%
>Zm01a10.y well B05 Zm1-2.y.z Run01 Cimarron 3.12 0 (65/2284)---- 2.85%
>Zm01a10.z well B17 Zm1-2.y.z Run01 Cimarron 3.12 85 (214/3636)---- 5.89%
>Zm01a11.z well A18 Zm1-2.y.z Run01 Cimarron 3.12 32 (161/1093)--- 14.73%
>Zm01a12.y well B06 Zm1-2.y.z Run01 Cimarron 3.12 624 (660/867)--- 76.12%
>Zm01a12.z well B18 Zm1-2.y.z Run01 Cimarron 3.12 32 (153/906)---16.88%
>Zm01b01.y well C01 Zm1-2.y.z Run01 Cimarron 3.12 566 (578/972)--- 59.47%
>Zm01b01.z well C13 Zm1-2.y.z Run01 Cimarron 3.12 504 (511/1188)---43.01%
>Zm01b02.y well D01 Zm1-2.y.z Run01 Cimarron 3.12 0 (125/2127)---- 5.87%
>Zm01b02.z well D13 Zm1-2.y.z Run01 Cimarron 3.12 108 (108/813)--- 13.28%
>Zm01b03.y well C02 Zm1-2.y.z Run01 Cimarron 3.12 477 (511/676)--- 75.59%
>Zm01b03.z well C14 Zm1-2.y.z Run01 Cimarron 3.12 277 (414/1567)--- 26.42%
>Zm01b04.y well D02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01b04.z well D14 Zm1-2.y.z Run01 Cimarron 3.12 728 (736/798)--- 92.23%
>Zm01b05.y well C03 Zm1-2.y.z Run01 Cimarron 3.12 241 (335/2593)--- 12.92
>Zm01b05.z well C15 Zm1-2.y.z Run01 Cimarron 3.12 142 (142/1457)--- 9.74%
>Zm01b06.y well D03 Zm1-2.y.z Run01 Cimarron 3.12 33 (90/3346)--- 2.69%
>Zm01b06.z well D15 Zm1-2.y.z Run01 Cimarron 3.12 768 (768/770)--- 99.74%
>Zm01b07.z well C16 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01b08.y well D04 Zm1-2.y.z Run01 Cimarron 3.12 587 (594/701)--- 84.74%
>Zm01b08.z well D16 Zm1-2.y.z Run01 Cimarron 3.12 312 (328/992)--- 33.06%
>Zm01b09.y well C05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01b09.z well C17 Zm1-2.y.z Run01 Cimarron 3.12 153 (161/3512)--- 4.58%
>Zm01b10.y well D05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01b10.z well D17 Zm1-2.y.z Run01 Cimarron 3.12 0 (104/1749)--- 5.95%
>Zm01b11.y well C06 Zm1-2.y.z Run01 Cimarron 3.12 156 (279/443)---62.97%

>Zm01b11.z well C18 Zm1-2.y.z Run01 Cimarron 3.12 260 (266/1008)--- 26.39%
>Zm01b12.y well D06 Zm1-2.y.z Run01 Cimarron 3.12 103 (103/587)--- 17.55%
>Zm01b12.z well D18 Zm1-2.y.z Run01 Cimarron 3.12 63 (114/597)--- 19.09%
>Zm01c01.y well E01 Zm1-2.y.z Run01 Cimarron 3.12 282 (282/2559)--- 11.02%
>Zm01c02.y well F01 Zm1-2.y.z Run01 Cimarron 3.12 672 (675/887)--- 76.10%
>Zm01c02.z well F13 Zm1-2.y.z Run01 Cimarron 3.12 158 (281/610)--- 46.07%
>Zm01c03.y well E02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c03.z well E14 Zm1-2.y.z Run01 Cimarron 3.12 321 (473/972)--- 48.66%
>Zm01c04.y well F02 Zm1-2.y.z Run01 Cimarron 3.12 573 (589/1001)--- 58.84%
>Zm01c04.z well F14 Zm1-2.y.z Run01 Cimarron 3.12 99 (120/1097)--- 10.93%
>Zm01c05.y well E03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c06.y well F03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c07.z well E16 Zm1-2.y.z Run01 Cimarron 3.12 0 (54/1074)--- 5.03%
>Zm01c08.z well F16 Zm1-2.y.z Run01 Cimarron 3.12 45 (67/3916)--- 1.71%
>Zm01c09.z well E17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c10.z well F17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c11.y well E06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01c12.z well F18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01d01.y well G01 Zm1-2.y.z Run01 Cimarron 3.12 376 (400/792) --- 50.50%
>Zm01d01.z well G13 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01d02.y well H01 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01d03.y well G02 Zm1-2.y.z Run01 Cimarron 3.12 563 (563/814)--- 69.17%
>Zm01d03.z well G14 Zm1-2.y.z Run01 Cimarron 3.12 376 (376/1235)--- 30.44%
>Zm01d04.y well H02 Zm1-2.y.z Run01 Cimarron 3.12 576 (635/991)--- 64.08%
>Zm01d04.z well H14 Zm1-2.y.z Run01 Cimarron 3.12 12 (65/2920)--- 22.26%
>Zm01d05.z well G15 Zm1-2.y.z Run01 Cimarron 3.12 427 (431/2101--- 20.51%
>Zm01d06.z well H15 Zm1-2.y.z Run01 Cimarron 3.12 0 (72/631)--- 11.41%
>Zm01d07.y well G04 Zm1-2.y.z Run01 Cimarron 3.12 521 (525/637)--- 82.42%
>Zm01d08.z well H16 Zm1-2.y.z Run01 Cimarron 3.12 171 (187/662)--- 28.22%
>Zm01d09.y well G05 Zm1-2.y.z Run01 Cimarron 3.12 13
>Zm01d09.z well G17 Zm1-2.y.z Run01 Cimarron 3.12 54 (88/1141)---- 7.71%
>Zm01d10.y well H05 Zm1-2.y.z Run01 Cimarron 3.12 230 (253/2846)--- 8.89%
>Zm01d10.z well H17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01d11.y well G06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01d11.z well G18 Zm1-2.y.z Run01 Cimarron 3.12 351 (354/618)---- 57.28%
>Zm01d12.z well H18 Zm1-2.y.z Run01 Cimarron 3.12 77 (104/3275)---- 3.18%
>Zm01e01.y well I01 Zm1-2.y.z Run01 Cimarron 3.12 467 (476/1127)——-42.23%
>Zm01e01.z well I13 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e02.y well J01 Zm1-2.y.z Run01 Cimarron 3.12 604 (619/942)--- 65.71%
>Zm01e02.z well J13 Zm1-2.y.z Run01 Cimarron 3.12 142 (153/690)--- 22.72%
>Zm01e03.z well I14 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e04.y well J02 Zm1-2.y.z Run01 Cimarron 3.12 797 (808/1015)--- 79.05%
>Zm01e04.z well J14 Zm1-2.y.z Run01 Cimarron 3.12 223 (235/768)--- 30.59%
>Zm01e06.y well J03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e06.z well J15 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e08.z well J16 Zm1-2.y.z Run01 Cimarron 3.12 0

>Zm01e09.y well I05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e09.z well I17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e10.z well J17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e11.z well I18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01e12.z well J18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f01.y well K01 Zm1-2.y.z Run01 Cimarron 3.12 315 (320/982)--- 32.59%
>Zm01f01.z well K13 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f02.y well L01 Zm1-2.y.z Run01 Cimarron 3.12 762 (762/1031)--- 73.91%
>Zm01f02.z well L13 Zm1-2.y.z Run01 Cimarron 3.12 496 (509/731)--- 69.63%
>Zm01f03.y well K02 Zm1-2.y.z Run01 Cimarron 3.12 912 (912/928)--- 98.27%
>Zm01f04.y well L02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f04.z well L14 Zm1-2.y.z Run01 Cimarron 3.12 112 (226/445)--- 50.78%
>Zm01f05.y well K03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f05.z well K15 Zm1-2.y.z Run01 Cimarron 3.12 156 (285/1968)--- 14.48%
>Zm01f06.z well L15 Zm1-2.y.z Run01 Cimarron 3.12 499 (512/767)--- 66.74%
>Zm01f07.y well K04 Zm1-2.y.z Run01 Cimarron 3.12 516 (557/848)--- 65.68%
>Zm01f07.z well K16 Zm1-2.y.z Run01 Cimarron 3.12 111 (117/128) --- 91.40%
>Zm01f08.y well L04 Zm1-2.y.z Run01 Cimarron 3.12 789 (797/807) ---98.76%
>Zm01f08.z well L16 Zm1-2.y.z Run01 Cimarron 3.12 451 (451/983)--- 45.88%
>Zm01f09.z well K17 Zm1-2.y.z Run01 Cimarron 3.12 414 (433/2349)--- 18.43%
>Zm01f10.y well L05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f10.z well L17 Zm1-2.y.z Run01 Cimarron 3.12 470 (470/1896)--- 24.79%
>Zm01f11.z well K18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f12.y well L06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01f12.z well L18 Zm1-2.y.z Run01 Cimarron 3.12 653 (653/1691)--- 38.61%
>Zm01g01.y well M01 Zm1-2.y.z Run01 Cimarron 3.12 728 (728/944)--- 77.11%
>Zm01g01.z well M13 Zm1-2.y.z Run01 Cimarron 3.12 527 (542/1126)--- 48.13%
>Zm01g02.y well N01 Zm1-2.y.z Run01 Cimarron 3.12 771 (779/1005)--- 77.51%
>Zm01g02.z well N13 Zm1-2.y.z Run01 Cimarron 3.12 329 (344/678)--- 50.74%
>Zm01g03.y well M02 Zm1-2.y.z Run01 Cimarron 3.12 273 (273/548)--- 49.82%
>Zm01g03.z well M14 Zm1-2.y.z Run01 Cimarron 3.12 553 (566/1683)--- 33.63%
>Zm01g04.y well N02 Zm1-2.y.z Run01 Cimarron 3.12 731 (749/849)--- 88.21%
>Zm01g04.z well N14 Zm1-2.y.z Run01 Cimarron 3.12 0 (97/2378)--- 4.07%
>Zm01g05.y well M03 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01g05.z well M15 Zm1-2.y.z Run01 Cimarron 3.12 413 (413/809)--- 51.05%
>Zm01g06.y well N03 Zm1-2.y.z Run01 Cimarron 3.12 642 (642/817)--- 78.58%
>Zm01g06.z well N15 Zm1-2.y.z Run01 Cimarron 3.12 636 (640/809)--- 79.11%
>Zm01g07.z well M16 Zm1-2.y.z Run01 Cimarron 3.12 661 (670/749)--- 89.41%
>Zm01g08.y well N04 Zm1-2.y.z Run01 Cimarron 3.12 13
>Zm01g08.z well N16 Zm1-2.y.z Run01 Cimarron 3.12 656 (695/763)--- 91.09%
>Zm01g09.y well M05 Zm1-2.y.z Run01 Cimarron 3.12 447 (562/679)--- 82.77%
>Zm01g09.z well M17 Zm1-2.y.z Run01 Cimarron 3.12 276 (524/1180)--- 44.41%
>Zm01g10.y well N05 Zm1-2.y.z Run01 Cimarron 3.12 784 (788/820)--- 96.1%
>Zm01g10.z well N17 Zm1-2.y.z Run01 Cimarron 3.12 430 (443/492)--- 90.04%
>Zm01g11.y well M06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01g11.z well M18 Zm1-2.y.z Run01 Cimarron 3.12 582 (583/1532)--- 38.06%

>Zm01g12.z well N18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h02.z well P13 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h03.y well O02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h03.z well O14 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h04.y well P02 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h04.z well P14 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h05.z well O15 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h06.z well P15 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h08.y well P04 Zm1-2.y.z Run01 Cimarron 3.12 3
>Zm01h08.z well P16 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h09.y well O05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h09.z well O17 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h10.y well P05 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h11.y well O06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h11.z well O18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h12.y well P06 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm01h12.z well P18 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02a01.z well A19 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02a02.y well B07 Zm1-2.y.z Run01 Cimarron 3.12 94
>Zm02a02.z well B19 Zm1-2.y.z Run01 Cimarron 3.12 72
>Zm02a03.y well A08 Zm1-2.y.z Run01 Cimarron 3.12 643 (644/691)--- 93.19%
>Zm02a03.z well A20 Zm1-2.y.z Run01 Cimarron 3.12 492 (492/2337)--- 21.05%
>Zm02a04.y well B08 Zm1-2.y.z Run01 Cimarron 3.12 537 (555/575)--- 96.52%
>Zm02a04.z well B20 Zm1-2.y.z Run01 Cimarron 3.12 402 (408/761)--- 53.62%
>Zm02a05.y well A09 Zm1-2.y.z Run01 Cimarron 3.12 702 (709/782)--- 90.66%
>Zm02a06.y well B09 Zm1-2.y.z Run01 Cimarron 3.12 392 (404/626)--- 94.84%
>Zm02a06.z well B21 Zm1-2.y.z Run01 Cimarron 3.12 528 (528/2361)--- 22.63%
>Zm02a07.y well A10 Zm1-2.y.z Run01 Cimarron 3.12 237 (246/1011)--- 24.33%
>Zm02a07.z well A22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02a08.y well B10 Zm1-2.y.z Run01 Cimarron 3.12 0 (228/1075)--- 21.21%
>Zm02a08.z well B22 Zm1-2.y.z Run01 Cimarron 3.12 402 (402/612)--- 65.69%
>Zm02a10.z well B23 Zm1-2.y.z Run01 Cimarron 3.12 499 (520/1974) ---26.34%
>Zm02a11.y well A12 Zm1-2.y.z Run01 Cimarron 3.12 661 (667/865)--- 77.11%
>Zm02a11.z well A24 Zm1-2.y.z Run01 Cimarron 3.12 469 (469/2401)--- 19.53%
>Zm02a12.y well B12 Zm1-2.y.z Run01 Cimarron 3.12 408 (413/581)--- 71.08%
>Zm02a12.z well B24 Zm1-2.y.z Run01 Cimarron 3.12 264 (271/638)--- 42.47%
>Zm02b01.y well C07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02b01.z well C19 Zm1-2.y.z Run01 Cimarron 3.12 422 (610/800)---- 76.25%
>Zm02b02.y well D07 Zm1-2.y.z Run01 Cimarron 3.12 547 (701/1105)--- 63.44%
>Zm02b02.z well D19 Zm1-2.y.z Run01 Cimarron 3.12 252 (252/791)--- 31.59%
>Zm02b03.y well C08 Zm1-2.y.z Run01 Cimarron 3.12 817 (817/865)--- 94.45%
>Zm02b03.z well C20 Zm1-2.y.z Run01 Cimarron 3.12 257 (273/565) – 48.31%
>Zm02b04.y well D08 Zm1-2.y.z Run01 Cimarron 3.12 658 (666/676)--- 98.52%
>Zm02b04.z well D20 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02b05.y well C09 Zm1-2.y.z Run01 Cimarron 3.12 723 (725/739)--- 98.11%
>Zm02b05.z well C21 Zm1-2.y.z Run01 Cimarron 3.12 202 (202/3107)--- 6.50%

>Zm02b06.y well D09 Zm1-2.y.z Run01 Cimarron 3.12 55 (152/1070)--- 14.20%
>Zm02b06.z well D21 Zm1-2.y.z Run01 Cimarron 3.12 287 (287/3037)--- 9.45%
>Zm02b07.y well C10 Zm1-2.y.z Run01 Cimarron 3.12 845 (861/992)--- 86.79%
>Zm02b07.z well C22 Zm1-2.y.z Run01 Cimarron 3.12 394 (394/885)--- 44.52%
>Zm02b08.y well D10 Zm1-2.y.z Run01 Cimarron 3.12 683 (688/1048)--- 65.65%
>Zm02b08.z well D22 Zm1-2.y.z Run01 Cimarron 3.12 637 (637/1962)---32.47%
>Zm02b09.y well C11 Zm1-2.y.z Run01 Cimarron 3.12 779 (785/835)--- 94.01%
>Zm02b09.z well C23 Zm1-2.y.z Run01 Cimarron 3.12 501 (501/864)--- 57.98%
>Zm02b10.z well D23 Zm1-2.y.z Run01 Cimarron 3.12 162 (288/665)--- 43.31%
>Zm02b11.y well C12 Zm1-2.y.z Run01 Cimarron 3.12 653 (653/729)--- 89.58%
>Zm02b11.z well C24 Zm1-2.y.z Run01 Cimarron 3.12 125 (126/2388)--- 5.28%
>Zm02b12.y well D12 Zm1-2.y.z Run01 Cimarron 3.12 709 (709/990)---- 71.61%
>Zm02b12.z well D24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c01.y well E07 Zm1-2.y.z Run01 Cimarron 3.12 209 (226/952)--- 23.74%
>Zm02c01.z well E19 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c02.y well F07 Zm1-2.y.z Run01 Cimarron 3.12 562 (580/601)---- 96.51%
>Zm02c02.z well F19 Zm1-2.y.z Run01 Cimarron 3.12 727 (763/801)---- 92.56%
>Zm02c03.y well E08 Zm1-2.y.z Run01 Cimarron 3.12 0 (223/2641)---8.44%
>Zm02c03.z well E20 Zm1-2.y.z Run01 Cimarron 3.12 345 (359/932)--- 38.52%
>Zm02c04.y well F08 Zm1-2.y.z Run01 Cimarron 3.12 577 (582/823)--- 70.71%
>Zm02c04.z well F20 Zm1-2.y.z Run01 Cimarron 3.12 671 (671/1037)--- 64.71%
>Zm02c05.y well E09 Zm1-2.y.z Run01 Cimarron 3.12 112 (243/3237)--- 7.51%
>Zm02c05.z well E21 Zm1-2.y.z Run01 Cimarron 3.12 341 (343/2543)--- 13.48%
>Zm02c06.y well F09 Zm1-2.y.z Run01 Cimarron 3.12 0 (142/1493)--- 9.51%
>Zm02c06.z well F21 Zm1-2.y.z Run01 Cimarron 3.12 307 (339/595)--- 56.97%
>Zm02c07.z well E22 Zm1-2.y.z Run01 Cimarron 3.12 508 (508/2108)--- 24.09%
>Zm02c08.y well F10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c08.z well F22 Zm1-2.y.z Run01 Cimarron 3.12 397 (397/849)--- 46.76%
>Zm02c09.y well E11 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c09.z well E23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c10.y well F11 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02c10.z well F23 Zm1-2.y.z Run01 Cimarron 3.12 471 (484/2577)--- 18.78%
>Zm02c11.y well E12 Zm1-2.y.z Run01 Cimarron 3.12 153 (278/2298)--- 12.09%
>Zm02c11.z well E24 Zm1-2.y.z Run01 Cimarron 3.12 65 (185/1527)--- 12.11%
>Zm02c12.z well F24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d01.y well G07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d01.z well G19 Zm1-2.y.z Run01 Cimarron 3.12 374 (388/809)--- 47.96%
>Zm02d02.y well H07 Zm1-2.y.z Run01 Cimarron 3.12 264 (264/2335)--- 11.31%
>Zm02d02.z well H19 Zm1-2.y.z Run01 Cimarron 3.12 41
>Zm02d03.y well G08 Zm1-2.y.z Run01 Cimarron 3.12 462 (463/964)--- 48.03%
>Zm02d03.z well G20 Zm1-2.y.z Run01 Cimarron 3.12 93
>Zm02d04.y well H08 Zm1-2.y.z Run01 Cimarron 3.12 462 (483/596)--- 81.04%
>Zm02d04.z well H20 Zm1-2.y.z Run01 Cimarron 3.12 197 (197/761)--- 25.88%
>Zm02d05.y well G09 Zm1-2.y.z Run01 Cimarron 3.12 350 (370/1823)--- 20.29%
>Zm02d05.z well G21 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d06.y well H09 Zm1-2.y.z Run01 Cimarron 3.12 495 (500/875)--- 57.12%

>Zm02d06.z well H21 Zm1-2.y.z Run01 Cimarron 3.12 293 (293/769)--- 38.01%
>Zm02d07.z well G22 Zm1-2.y.z Run01 Cimarron 3.12 312 (312/2172)---14.36%
>Zm02d08.z well H22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d09.z well G23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d10.z well H23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d11.y well G12 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d11.z well G24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d12.y well H12 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02d12.z well H24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e01.y well I07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e01.z well I19 Zm1-2.y.z Run01 Cimarron 3.12 487 (522/1789)--- 29.18%
>Zm02e02.z well J19 Zm1-2.y.z Run01 Cimarron 3.12 453 (453/1729)--- 26.2%
>Zm02e03.y well I08 Zm1-2.y.z Run01 Cimarron 3.12 32 (242/473)--- 51.16%
>Zm02e03.z well I20 Zm1-2.y.z Run01 Cimarron 3.12 361 (375/756)--- 49.60
>Zm02e04.z well J20 Zm1-2.y.z Run01 Cimarron 3.12 391 (396/762)--- 51.97%
>Zm02e05.y well I09 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e05.z well I21 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e06.y well J09 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e07.y well I10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e07.z well I22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e08.y well J10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e09.z well I23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e10.y well J11 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e10.z well J23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e11.z well I24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02e12.z well J24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02f01.z well K19 Zm1-2.y.z Run01 Cimarron 3.12 686 (718/813) --- 88.31%
>Zm02f02.y well L07 Zm1-2.y.z Run01 Cimarron 3.12 344 (358/681)--- 52.57%
>Zm02f02.z well L19 Zm1-2.y.z Run01 Cimarron 3.12 739 (739/1124)--- 65.75%
>Zm02f03.y well K08 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02f03.z well K20 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02f04.z well L20 Zm1-2.y.z Run01 Cimarron 3.12 433 (446/738)--- 60.43%
>Zm02f05.y well K09 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02f05.z well K21 Zm1-2.y.z Run01 Cimarron 3.12 285 (300/3035)--- 9.88%
>Zm02f06.y well L09 Zm1-2.y.z Run01 Cimarron 3.12 0 (145/1216)--- 11.92%
>Zm02f06.z well L21 Zm1-2.y.z Run01 Cimarron 3.12 0 (111/1769)--- 6.27%
>Zm02f08.y well L10 Zm1-2.y.z Run01 Cimarron 3.12 418 (418/773)---54.08%
>Zm02f08.z well L22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02f09.y well K11 Zm1-2.y.z Run01 Cimarron 3.12 532 (536/773)--- 69.34%
>Zm02f09.z well K23 Zm1-2.y.z Run01 Cimarron 3.12 506 (518/820)--- 63.17%
>Zm02f10.y well L11 Zm1-2.y.z Run01 Cimarron 3.12 633 (635/1033)--- 61.47%
>Zm02f10.z well L23 Zm1-2.y.z Run01 Cimarron 3.12 241 (241/680)--- 35.44%
>Zm02f11.y well K12 Zm1-2.y.z Run01 Cimarron 3.12 726 (726/728) --- 99.72%
>Zm02f11.z well K24 Zm1-2.y.z Run01 Cimarron 3.12 349 (349/1526)--- 22.87%
>Zm02f12.y well L12 Zm1-2.y.z Run01 Cimarron 3.12 185 (247/598)--- 41.30%
>Zm02f12.z well L24 Zm1-2.y.z Run01 Cimarron 3.12 96

>Zm02g01.y well M07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g01.z well M19 Zm1-2.y.z Run01 Cimarron 3.12 763 (763/1029)--- 74.15%
>Zm02g02.z well N19 Zm1-2.y.z Run01 Cimarron 3.12 64
>Zm02g03.y well M08 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g03.z well M20 Zm1-2.y.z Run01 Cimarron 3.12 682 (692/733)--- 94.40%
>Zm02g04.y well N08 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g04.z well N20 Zm1-2.y.z Run01 Cimarron 3.12 291 (309/711)--- 43.46%
>Zm02g05.y well M09 Zm1-2.y.z Run01 Cimarron 3.12 0 (138/1806)---7.64%
>Zm02g05.z well M21 Zm1-2.y.z Run01 Cimarron 3.12 289 (291/1056)--- 27.56%
>Zm02g06.y well N09 Zm1-2.y.z Run01 Cimarron 3.12 0 (154/2005)--- 7.68%
>Zm02g06.z well N21 Zm1-2.y.z Run01 Cimarron 3.12 251 (265/3025)--- 8.76%
>Zm02g07.y well M10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g07.z well M22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g08.y well N10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g08.z well N22 Zm1-2.y.z Run01 Cimarron 3.12 4
>Zm02g09.z well M23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g11.y well M12 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g11.z well M24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02g12.y well N12 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h01.y well O07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h01.z well O19 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h02.y well P07 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h02.z well P19 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h03.z well O20 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h04.z well P20 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h05.z well O21 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h06.z well P21 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h07.y well O10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h07.z well O22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h08.y well P10 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h08.z well P22 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h09.y well O11 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h09.z well O23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h10.z well P23 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h11.y well O12 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h11.z well O24 Zm1-2.y.z Run01 Cimarron 3.12 0
>Zm02h12.y well P12 Zm1-2.y.z Run01 Cimarron 3.12 0

Appendix-IV

Microsatellite repeats identified in the *ber* clone sequences with SSRIT

Sequence	Motif	No. of repeats	SSR start	SSR end	Seq. length
Dimer repeats (>5 repeats)					
Zm01a05.z-1	GA	5	449	458	846
Zm01a12.y-1	TA	5	153	162	660
Zm01e04.y-1	CT	14	313	340	808
Zm01g05.z-1	AG	8	201	216	413
Zm02b06.y-1	CA	5	86	95	152
Zm02c03.y-1	CA	5	43	52	223
Zm02c03.z-1	TA	7	195	208	359
Trimer (>4 repeats)					
Zm02e02.z-1	GAG	4	261	272	453
Zm02a08.y-1	GTA	5	212	226	228
Tetramer (>3 repeats)					
Zm01c02.y-1	TCTT	3	229	240	675
Zm01d03.y-1	AATT	3	316	327	563
Zm01d03.y-2	AATT	3	346	357	563
Zm01d03.z-2	TAAT	3	266	277	376
Zm01d03.z-3	AATT	3	298	309	376
Zm02a11.y-1	AATT	3	583	594	667
Zm02a11.y-2	AATT	3	614	625	667
Zm02a11.z-1	TTAA	3	208	219	469
Zm02a11.z-2	AATT	3	240	251	469
Zm02c03.z-4	ATAC	3	206	217	359
Zm02c07.z-5	ACCA	3	181	192	508
Pentamer (>2repeats)					
Zm01a05.z-44	AAAGA	2	468	477	846
Zm01a06.z-31	CCAAG	2	458	467	669
Zm01b01.y-25	CTTGG	2	464	473	578
Zm01b01.z-17	CCAAG	2	405	414	511
Zm01b03.z-49	CCTAA	2	225	234	414
Zm01b04.z-39	TTCCT	2	236	245	736
Zm01b06.z-41	AATGG	2	216	225	768

Zm01b06.z-42	TTCCT	2	525	534	768
Zm01b08.y-41	ATCTA	2	173	182	594
Zm01b08.z-17	CTTAG	2	287	296	328
Zm01c04.y-35	CGTTT	2	461	470	599
Zm01d05.z-19	TCTGT	2	256	265	431
Zm01f02.y-35	GGTAA	2	176	185	772
Zm01f03.y-49	AACTG	2	237	246	912
Zm01f07.y-29	AAGCT	2	375	384	557
Zm01f07.y-30	TGCTT	2	389	398	557
Zm01f08.y-50	CTCGA	2	743	752	797
Zm01f08.y-51	TCTTG	2	774	783	797
Zm01f10.z-25	TTGCG	2	395	404	470
Zm01g02.y-39	ATCTA	2	361	370	779
Zm01g02.y-40	CCTTT	2	734	743	779
Zm01g02.z-16	TAGAT	2	115	124	344
Zm01g10.y-35	GGCGG	2	97	106	788
Zm01g10.y-36	TTGTT	2	302	311	788
Zm01g10.y-37	AAGGT	2	583	592	788
Zm01g10.y-38	TGACA	2	727	736	788
Zm01g10.z-21	ACCTT	2	413	422	443
Zm01g11.z-33	GAATG	2	527	536	583
Zm01h09.y-3	GCCGG	2	34	43	47
Zm02a03.y-38	CAAAA	2	219	228	644
Zm02a11.y-30	CCAAG	2	172	181	667
Zm02a11.y-31	AGGAA	2	637	646	667
Zm02a11.z-18	TTTCC	2	188	197	469
Zm02b01.z-23	GTTAA	2	18	27	610
Zm02b02.y-37	AGCTT	2	297	306	701
Zm02b03.y-31	AATTA	2	242	251	817
Zm02b03.y-32	AATTA	2	314	323	817
Zm02b03.y-33	AATTA	2	373	382	817
Zm02b03.y-34	TTTAT	2	484	493	817
Zm02b05.y-39	GGCGG	2	91	100	725
Zm02b06.y-11	GCCCA	2	78	87	152
Zm02b08.y-33	GGAGA	2	348	357	688
Zm02b12.y-39	TCTTT	2	338	347	709
Zm02c04.z-40	TTGCG	2	718	727	734

Zm02c10.z-14	GAAAC	2	203	212	484
Zm02c11.y-14	TTTTC	2	150	159	275
Zm02d04.y-32	TTTGA	2	414	423	483
Zm02e02.z-19	TTGAT	2	162	171	453
Zm02e03.z-20	CTTTG	2	153	162	375
Zm02e04.z-20	TATTT	2	226	235	396
Zm02f08.y-26	GCTAT	2	49	58	418
Zm02f09.y-27	CGGGC	2	97	106	536
Zm02f09.y-28	AAACA	2	250	259	536
Zm02f09.z-29	TTGTT	2	361	370	519
Zm02f09.z-30	AAACT	2	469	478	519
Zm02f11.y-44	AAAAC	2	148	157	728

LIST OF PUBLICATIONS

Research paper in Journals

1. **Devanshi**, AK Singh, Pankaj Sharma, B Singh, R Singh and NK Singh. **2007**. Molecular profiling and genetic relationship study among ber (*Ziziiphus* sp.) genotypes using RAPD markers. *Indian J. Genet. Pl. Breed.* 67 (2) 121-127.

2. AK Singh, **Devanshi**, Pankaj Sharma, R Singh B. Singh, K R Koundal and N. K. Singh **2007**. Assessment of genetic diversity in *ziziphus* species using inter-simple sequence repeat (ISSR) markers. *Indian J. Pl. Biochem & Biotech.* 96 (1): 35-40.

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Assessment of Genetic Diversity in *Ziziphus mauritiana* Using Inter-Simple Sequence Repeat Markers

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Genetic diversity among 47 *ber* accessions belonging to cultivated species (*Ziziphus mauritiana* Lam) and one wild accession of *Ziziphus nummularia* (Burm F) Willed was investigated using Inter-Simple Sequence Repeat (ISSR) markers. A total of 167 amplification products were detected with 18 ISSR primers of which 152 (89.96%) were polymorphic. Most of the primers that produced distinct bands (14 primers out of 18) contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more polymorphic bands. Genetic similarity ranging from 43.07% to 90.30% suggested that the 48 *Ziziphus* genotypes used in the study were divergent. Cluster analysis based on UPGMA method and Bootstrap analysis separated all the 48 genotypes in four distinct clusters. The present study has successfully distinguished morphologically similar genotypes that emphasize the use of molecular markers to the taxonomists. Morphologically similar but genetically distinct genotypes, identified using ISSR markers could be potential sources for genotype identification and to resolve controversies over misnomination of *ber* genotypes. Present study is the first report on the exploitation of ISSR markers in *ber* for genetic diversity analysis.

Key words: *Ziziphus mauritiana*, genetic diversity, cluster analysis, ISSR, UPGMA.

The genus *Ziziphus* commonly known as *ber* belongs to the buckthorn family Rhamnaceae. It is a genus of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (1). Some species like *Z. mauritiana* and *Z. jujuba*, occur in nearly every continent, whereas, other species like *Z. nummularia*, *Z. spina-christi* and *Z. mucronata* are restricted in their distribution. These species can grow either as trees and shrubs (*Z. mauritiana, Z. rotundifolia, Z. jujube and Z. mucronata*) or exclusively as small shrubs or bushes (*Z. nummularia, Z. lotus, Z. spina-christi and Z. obtusifolia*). The Indian *ber*, *Ziziphus mauritiana* Lam, a beautiful evergreen tree is an example of extremely drought-hardy species and is a dominant component of the natural vegetation in the Indian desert. The scions of improved varieties are routinely grafted on to the rootstocks of wild species to provide a reasonable cash crop on lands unsuitable for other forms of cultivation (2). Improved Indian cultivars like Gola and Seb have been introduced in Israel and some countries of Africa, where they have been grafted onto native rootstocks of *Z. spina-christi* and *Z. abyssinica*, respectively (3). The same technique was successfully used in Zimbabwe to propagate high-quality Indian selections on the popular *Z. nummularia* rootstocks (4).

The wide geographical and climatic distribution is indicative of the fact that there exists a tremendous genetic diversity in *ber* which needs to be identified and catalogued. South and Southeast Asia is the centre of both evolution and distribution of the genus *Ziziphus* (5). Diversity among and within breeding material and elite germplasm is key to successful breeding programs (6). A large number of methodologies exist for the assessment of genetic diversity in plant species. A combination of morphological traits and protein profiling methods such as isozymes (7), allozymes (8) and seed storage proteins (9) has conventionally been applied. However, such traits are influenced by environmental factors as well as the developmental stage of the plants. Hence, the result elucidated based on such studies do not provide a true measure of the genetic diversity. Molecular markers based on PCR method offer several advantages over the sole use of conventional morphological markers.

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Abbreviations: ISSR- Inter Simple Sequence Repeat; UPGMA- Unweighted Paired Group Method of Arithmetic averages; RAPD- Random Amplified Polymorphic DNA; AFLP- Amplified Fragment Length Polymorphism.

The ISSR markers are very useful tool to detect genetic polymorphism (10). ISSR markers are inexpensive and readily adaptable technique for routine germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes (11) and construction of genetic linkage maps (12). Although genetic diversity analysis and cultivar identification by RAPD and ISSR markers have been performed in many fruits (13-15), its application in *Ziziphus* species and its relatives (with exception of *Z. celata*) has not been carried out (16). AFLP has been used to document genetic diversity in limited accessions of *Ziziphus mauritiana* and *Z. nummularia* (17). Long juvenile phase of *Ziziphus* (up to 7 years) would make DNA markers an extremely useful tool for the identification of cultivars during propagation and growth. Cultivar identification using molecular markers would also aid in the management of germplasm collections of *Ziziphus*, as authenticity of many *ber* (*Z. mauritiana*) cultivars is unclear and the subject of some controversy. Although there exists a high level of morphological variability but the similar genotype is known by different names in different regions of cultivation, it is possibly due to influence of environment on morphology (18) and therefore morphological appearances are not reliable measure of diversity among *ber* genotypes. The genotypes of *ber* used in the present study represent only a subset of the existing natural variation in the species (*Ziziphus mauritiana*) and to date no systematic attempt has been made to understand the level of genetic variation that is of utmost need for designing further breeding strategies in this fruit crop. Therefore, the present study using ISSR markers aimed to asses the genetic diversity among *ber* genotypes grown in different geographical region of India.

Materials and Methods

Plant material and DNA Isolation — Leaf sample of forty-seven accessions of *ber* representing *Ziziphus mauritiana* and one accession of *Z. nummularia* already collected from different states of India and maintained in the orchard of Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi, India were used in present investigation (Table 1). Young leaves were collected from single tree of each cultivar and frozen in liquid nitrogen immediately. Leaf samples were stored at -80 °C till DNA was isolated. Total genomic DNA was extracted from green leaves using the cetyltrimethyl ammonium bromide (CTAB) method with some

Table 1. *Ber* genotypes analysed for diversity analysis using ISSR markers

Sl No.	Accessions	Area(s) of cultivation
Z. mauritiana Lam genotypes		
1	Sindhura	Haryana
2	Seb	Haryana
3	Vilayati	Haryana
4	Ilaichi	Uttar Pradesh
5	Gola	Rajasthan / Haryana
6	Muria Mehrora	Uttar Pradesh
7	Katha	Punjab
8	Thornless	Uttar Pradesh
9	Sanauri	Punjab
10	Banarasi Kadaka	Uttar Pradesh
11	Chhuahara	Rajasthan/Punjab
12	Narma	Delhi
13	Chonchal	Punjab
14	Bagwadi	Rajasthan
15	Pownda	Punjab
16	Sindhura Narwal	Haryana
17	Kala Gola	Haryana
18	Tass Bataso	Uttar Pradesh
19	Tikadi	Rajasthan
20	Dandan	Rajasthan
21	Umaran	Punjab
22	Lakhan	Punjab
23	Katha Bombay	Gujarat
24	Bawal Selection -2	Haryana
25	Pathan	Punjab
26	Akhrota	Punjab/Haryana
27	Rohtak Safeda	Haryana
28	Sanauri-3	Punjab
29	Jogia	Uttar Pradesh
30	Desi Alwar	Rajasthan
31	Nazuk	Rajasthan
32	Hesang Tsaon	Uttar Pradesh
33	No-A	Haryana/Gujrat
34	Kathi	Punjab/Gujrat/Rajasthan
35	Kaithali	Haryana
36	Noki	Punjab
37	Popular Gola	Delhi
38	Kheera	Uttar Pradesh
39	Sua	Haryana
40	Katha Rajasthan	Rajasthan
41	Reshmi	Punjab
42	Wild collection	Delhi
43	Govind Garh Selection	Haryana
44	Kishmish	Uttar Pradesh
45	Bawal Selection-1	Bawal, Haryana
46	Zg-3	Haryana
47	Ilaichi Jhajhar	Haryana
Z. nummularia (Burm F) Willed		
48	*Z. nummularia*	Uttar Pradesh

modifications (19). DNA was quantified in a TKO 100 Fluorometer (Hoefer, San Fransisco, CA).

Primers and PCR amplification conditions — Forty ISSR primers (15-16mer oligos, UBC: University of British, Columbia) were synthesized (Microsynth Gmbh, Switzerland) and were tested for PCR amplification on 12 randomly selected genotypes to identify primers that were giving good, scorable and polymorphic amplification products. In order to estimate experimental reproducibility, two independent amplifications were carried out for the selected primers over the same set of genotypes and the primers that showed a clear and reproducible band pattern were chosen for further study on 48 genotypes of *ber* (Table 2). Amplifications were carried out with 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.1% Triton X-100, 2% formamide, 200 nM primer, 1 unit of *Taq* DNA polymerase (Operon Technologies, Almada, CA, USA) and 25 ng of genomic DNA. Amplifications were carried out using a 96 thermal cycler (Perkin-Elmer, USA) programmed for 35 cycles as follows: an initial denatuartion was for 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C and final extension for 7 min at 72 °C. The amplification products were stored at 4 °C until loading. PCR-products were resolved at 60 Volts for 3 h on 1.6% agarose gel prepared in 1x TBE buffer. Gel was photographed using Gel-Documentation system (Gel Doc Mega, Biosystematica, UK).

Data analysis — The band profiles of each gel were scored visually and recorded as presence (1) or absence (0) of bands and binary quantitative data matrix was constructed. A pair-wise difference matrix between genotypes was determined using Jaccard Similarity coefficient (20). Data analysis was performed using the NTSYS-pc (Numerical Taxonomic System, 21) version 2.11 computer programme package and Winboot software. A dendrogram was constricted by UPGMA method to measure the resulting phenetic groups and the original matrix was bootstrapped 1000 times by employing Winboot to group the genotypes into discrete clusters.

The ability of a primer or technique to distinguish between large numbers of genotypes, i.e. Resolving Power of the primer (Rp) of selected ISSR primers were determined as described by Prevost and Wilkinson (22).

Table 2. ISSR primers along with their sequences and some characteristics of amplification products in the *ber* genotypes

UBC[1] Primer	Sequence (5′ - 3′)	TNB	NPB	P%	Rp	PIC
808	AGAGAGAGAGAGAGAGAGC	11	9	81.82	4.04	0.34
809	AGAGAGAGAGAGAGAGG	11	10	90.91	4.75	0.37
814	CTCTCTCTCTCTCTCTA	10	10	100.00	4.54	0.42
825	ACACACACACACACACT	9	6	66.67	2.34	0.23
829	TGTGTGTGTGTGTGTGC	12	10	83.33	4.21	0.51
840	GAGAGAGAGAGAGAGACTT	10	10	100.00	2.50	0.40
841	GAGAGAGAGAGAGAGACTC	10	10	100.00	5.29	0.53
848	CACACACACACACACAAGG	7	7	100.00	3.67	0.44
850	GTGTGTGTGTGTGTGTCTC	8	8	100.00	4.71	0.64
854	TCTCTCTCTCTCTCTCAGG	9	9	100.00	2.50	0.38
855	ACACACACACACACACCTT	10	9	90.00	3.79	0.60
856	ACACACACACACACACCTA	4	4	100.00	3.67	0.36
876	GATAGATAGACAGACA	9	9	100.00	3.71	0.36
880	GGAGAGGAGAGGAGA	9	7	77.78	5.63	0.54
889	AGTCGTAGTACACACACACACAC	7	4	57.14	2.04	0.24
890	ACGACTACGGTGTGTGTTTGTGT	9	9	100.00	1.00	0.56
894	TGGTAGCTCTTGTCAGGCAC	12	11	91.67	5.17	0.43
900	ACTTCCCCACAGGTTAACACA	10	8	80.00	2.63	0.27
	Total	167	152	-	66.19	7.62
	Average	9.28	8.44	89.96	3.68	0.42

*TNB= Total number of bands; NPB= Number of polymorphic bands; P%= Polymorphism percentage; Rp= Resolving power, and PIC= Polymorphic information content. [1]UBC: University of British, Columbia

The Polymorphism Information Content (PIC) expresses the discriminating power of the locus taking into account not only the number of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus, expressed as: PIC = 1- Σp_i^2 as suggested by Lynch and Walsh (23).

Results and Discussion

Initial screening of 40 ISSR primers on 12 genotypes showed that 22 of these were monomorphic. These 22 monomorphic primers were tested on the remaining 36 genotypes in 3 bulks of 12 genotypes each to check for any polymorphism. The bulk samples were prepared by pooling equal amount of purified genomic DNA from three different sets of 12 genotypes and finally the 18 polymorphic primers were used to analyse the diversity among 48 genotypes. Details regarding the polymorphic primers, the number and size of the polymorphic fragments revealed by each primer are presented in Table 2. The primers amplified 4 fragments with UBC-856 and 12 fragments with UBC-829 and UBC-894 (Table 2). Only reproducible fragments were considered for data analysis. The ISSR profile generated with primer UBC-855 is shown in Fig.1. A total of 167 scorable fragments were yielded by the 18 polymorphic primers with an average of 9.28 bands per primer ranging between 250-2500 bp. One hundred and fifty two fragments (89.96%) with an average of 8.44 per primer were polymorphic. Among the 18 polymorphic primers, 9 primers showed 100% polymorphic bands (Table 2). Out of 18 primers used, 14 were anchored; of which 12 were 3′-anchored and 2 were 5′ -anchored. Most of the primers that produced polymorphic bands (14 primers out of 18) contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more number of bands.

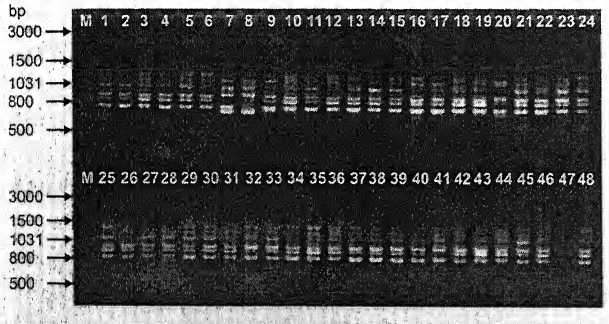

Fig. 1. ISSR profiles of 48 *ber* genotypes generated with primer UBC-855. M: 100bp DNA ladder, Lanes 1-48: correspond to *ber* genotypes listed in Table 1.

Resolving power (Rp) for each primer was calculated and it ranged from 1.0 (UBC-890) to 5.63 (UBC-880). The other primers with high Rp values were UBC-841 (5.29) and UBC-894 (5.17). The primers with high Rp values are able to distinguish among most of the *ber* genotypes (Table 2). Polymorphism information content (PIC) ranged from 0.23 (UBC-825) to 0.64 (UBC-850) with high PIC values for UBC-855 (0.60) and UBC-890 (0.56).

Genetic similarity between 48 accessions based on Jaccard coefficient ranged from 43.07% (between Katha and Seb) to 90.3% (between Narma and Banarasi Karaka). Dendrogram based on genetic similarity values were constructed to reveal similarities between varieties and later it was bootstrapped (1000 replications) to confirm the grouping (Fig. 2). The bootstrapped method showed that all the genotypes (except Tikadi and Sindhura) grouped into four clusters (Fig. 2). Not much variation in the dendrogram was observed between UPGMA and bootstrapped version. Dendrogram showed highest similarity between Narma and Banarasi Karaka (90.3%). Cluster I contained 2 genotypes viz., Katha and Thornless with 70.14% genetic similarity, cluster II with Rohtak safeda and Bawal Sel-2 and the cluster IV was consisted of five genotypes Seb, Chhuhara, No-A, Sindhura Narwal and Sanauri. Cluster III was the largest with 37 out of 48 genotypes subgrouped into five subclusters (Fig. 2). In subcluster III-D the wild accession of *Z. nummularia* grouped with Bagwadi, however, the grouping was not significant as revealed by bootstrap analysis. In our earlier study on AFLP analysis of *ber* (17), we have reported grouping of all the wild accessions of *Z. nummularia* together into a different sub-cluster away from *Z. mauritiana*. This is because AFLP primers amplified approximately six times more number of loci (952 loci) than the present ISSR analysis (167 loci). The present study using ISSR markers reliably distinguishes morphologically similar genotypes e.g. based on morphological traits Katha, Katha Bombay, Katha Rajasthan and Umran have been reported to be different names of the same genotype in different regions (18). However, in the present investigation we found that these four genotypes were genetically different thus shared different clusters. The genotypes Gola, Akhrota, Nazuk and Seb were earlier reported to be different morphologically although with insignificant differences but in the present study the earlier three genotypes grouped together in subcluster III-C, whereas, Seb shared 72.41% (57.3% of the 1000 replications) genetic similarity with the genotype

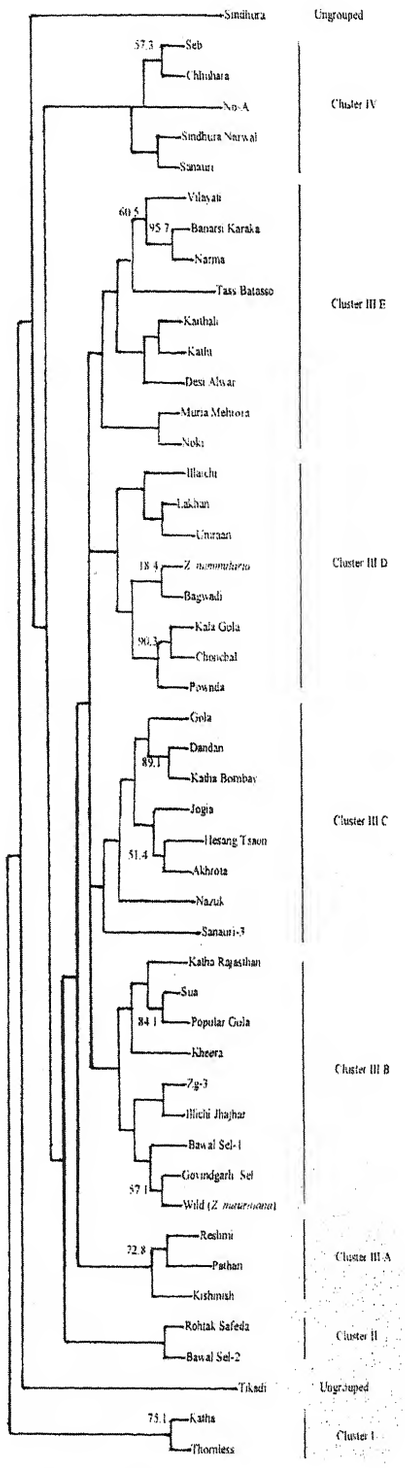

Fig. 2. Dendrogram generated through bootstrap analysis for 18 ISSR primers showing genetic relationships among the 48 *ber* genotypes. Names of the genotypes are given on the termini of branches. Numbers on the nodes are bootstrap values with 1000 replication.

Chhuhara. The genotypes Kala Gola, Popular Gola and Gola that are characteristically round in shape fell into different subclusters of cluster III sharing 76.03% genetic similarity between Popular Gola and Gola, 74.15% between Kala Gola and Gola, and 70.00% between Kala Gola and Popular Gola. Based on morphology, two genotypes Chhuhara and Reshmi are reported to be exactly same (18, 24) but based on the present study they share only 62.50% of genetic similarity and fall into different subclusters, IV and III-A, respectively. The results suggest that ISSR markers are able to genetically differentiate phenotypically similar genotypes. The present study has revealed a broad genetic base in *Ziziphus species* and suggests the taxonomist to review and resolve the mis-nomination of *ber* genotypes based not only morphologically but also using molecular markers. This study would help the consideration of molecular fingerprinting for germplasm conservation, rectification, purification and the identification of genotypes.

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Molecular profiling and genetic relationship among *ber* (*Ziziphus* sp.) genotypes using RAPD markers

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Abstract

Genetic relationship study was performed with RAPD markers among 50 *ber* genotypes representing *Z. mauritiana*, *Z. nummularia* and *Z. spina-christi*. Out of 120 primers initially tested, 46 were highly reproducible and generated 368 RAPD markers with 86.2% polymorphism (316 polymorphic bands). The number of amplification product per primer ranged from 2 (OPF-9) to 17 (OPD-3) with an average of 8 bands per primer. The resolving power (Rp) for different primers ranged from 0.48 (OPE-4) to 9.37 (OPD-3) and polymorphic information content (PIC) ranged from 0.12 (OPD-20) to 0.82 (OPE-9). Nineteen primers distinguished at least one genotype that would prove to be highly useful for identification of genotype and designing future breeding strategy. Genetic relationships between the accessions were established based on Jacquard's similarity coefficient and it ranged from 26.3% to 78.9% suggesting that the *Ziziphus* germplasm is genetically diverse. UPGMA cluster analysis generated dendrogram with six clusters separating two wild genotypes, *Z. nummularia* (collection 1) and *Z. spina-christi* from rest of the genotypes. Cluster-I and II comprised of two genotypes each, whereas, the biggest cluster, cluster VI comprised of 20 out of remaining 44 genotypes. In cluster II to cluster-VI, genotypes were separated from each other at different similarity levels in successive branching. The present study has proved that *ber* genotype earlier reported to be similar based on morphology are genetically different. The degree of genetic variation detected in *Ziziphus* species with RAPD analysis in the present study suggests that it is an efficient marker technology for delineating genetic relationships among genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategy for conservation and improvement programmes.

Key words: *Ziziphus* sp., genetic diversity, PIC, resolving power

Introduction

Ber (*Ziziphus mauritiana* L.) is cultivated all over the arid parts of Indian subcontinent. The tree is endowed with a high degree of edaphic and climatic adaptability and plays a major role in preventing soil erosion and desertification [1]. Its fresh fruits are rich in vitamins mainly C, A and B-complex [1]. South and Southeast Asia is considered as the centre for both evolution and distribution of the genus *Ziziphus* [2].

To assess genetic diversity in different plant species a large number of methodologies have been reported such as isozymes [3], allozymes [4] and seed storage proteins [5]. However, such traits are influenced by environmental factors and the developmental stage of the plant. Hence, the results elucidated based on such studies do not provide a true measure of the genetic diversity. Molecular markers based on Polymerase Chain Reaction (PCR) method offer several advantages over the conventional morphological markers. Random Amplified Polymorphic DNA (RAPD) uses arbitrary 10-base primers to amplify the random portions of the genome [6]. The fragments produced are easily visualized on ethidium bromide stained gel and polymorphism can be detected between the amplified products of different individuals. Although genetic diversity analysis and cultivar identification by RAPD and other molecular markers have been performed in many fruits [7, 8], its application in *Ziziphus* species and its relatives with exception of *Z. celata* [9] has not been carried out. Long juvenile phase of *Ziziphus* (up to 7 years) would make DNA markers an extremely useful tool for the identification of cultivars during propagation and growth. Cultivar identification using molecular markers would also aid in the management of germplasm collections of *Ziziphus*, as authenticity of many *ber* (*Z. mauritiana*) cultivars is unclear and the subject of some controversy. Moreover, because of the existence of a high level of morphological variability, morphological data can lead to ambiguous interpretations. We report here the RAPD markers to evaluate the genetic relationship among the 47 cultivars belonging to *Z. mauritiana*, two wild collections of *Z. nummularia* (designated as collection 1 and 2) and a single collection of *Z. spina-christi*. To our knowledge,

this is the first report on genetic diversity analysis of Indian *ber* using RAPD markers.

Materials and Methods

Plant material : Juvenile leaves of fifty genotypes (Table 1) were harvested in the month of May from single tree. The germplasm was maintained at Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi and Central Institute for Tropical Horticulture, Bikaner, India. Out of these 50 genotypes, *Z. nummularia* collection-2 was taken from Uttar Pradesh. The harvested leaves were immediately stored at –80°C until total genomic DNA was isolated using Cetyl-trimethyl Ammonium Bromide (CTAB) protocol [10] by adding PolyVinyl-Pyrrolidone (PVP).

Table 1. *Ziziphus* genotypes used for DNA fingerprinting and genetic relationship study

Species	Genotypes	No. of genotypes	Main areas of cultivation
Z. mauritiana Lam.	Sindhura, Seb, Vilayati, Sindhura Narwal, Kala Gola, Bawal Selection-1, Bawal Sel-2, Zg-3, Ilaichi Jhajhar, Rohtak Safeda, Kaithali, Sua, Govind Garh Selection	13	Haryana
	Katha, Sanauri, Chonchal, Pownda, Umaran, Lakhan, Pathan, Sanauri-3, Noki, Reshmi	10	Punjab
	Ilaichi, Muria Mehrara, Thornless, Banarasi Kadaka, Tass Bataso, Kishmish, Jogia, Hesang Tsaon, Kheera	9	Uttar Pradesh
	Desi Alwar, Nazuk, Bagwadi, Tikadi, Dandan, Katha Rajasthan	6	Rajasthan
	Narma, Popular Gola	2	Delhi
	Katha Bombay	1	Gujarat
	Gola	1	Rajasthan/Haryana
	Akhrota	1	Punjab/Haryana
	Chhuhara	1	Rajasthan/Punjab
	No-A	1	Haryana/Gujrat
	Kathi	1	Punjab/Gujrat/Rajsthan
	Wild collection	1	Delhi
Z. spina-christi Lam.	*Z. spina-christi*	1	Wild
Z. nummularia (Burm.f.) Willed.	*Z. nummularia* Collection 1	1	Wild
	Z. nummularia Collection 2	1	Wild (collected from Uttar Pradesh)

Primer screening : One hundred and twenty RAPD primers (A, B, C, D, E, F series) from Operon Technology (Alameda, California, USA) were tested on three cultivars to identify primers that were giving good, scorable and polymorphic products. In order to check the reproducibility, two independent amplifications were carried out and finally 46 RAPD primers that showed clear and reproducible bands were chosen for further study.

PCR analysis and gel electrophoresis : PCR amplification was performed in a 25µl reaction volume, containing 1X reaction buffer (10mM Tris HCl pH 8), 1.5mM $MgCl_2$, 200µM of each dNTP, 1 unit of Taq polymerase, 30µM of primer and approximately 25ng genomic DNA template. Each genotype primer combination was amplified twice to check the reproducibility. Pre-screened primers were selected for the study of genetic relationships. The thermocycler (Perkin Elmer 480) was programmed for an initial step of 4 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. A final extension step at 72°C was programmed for 7 minutes. The amplified products were stored at 4°C until loading.

Amplification products were separated on 1.4% agarose gels with 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8.0) at 3 V/cm for 4 hour. A 1 kb DNA ladder (MBI, Fermentas, USA) was used as a molecular size standard. Agarose gel was stained with 0.5µg/ml ethidium bromide solution and visualized under ultraviolet light and photographed with gel documentation.

Data analysis : DNA bands in the profile were scored visually and recorded as present (1) or absent (0) and the binary qualitative data matrix was constructed. Data analysis was performed using the NTSYS-pc software (version 2.1). The genetic similarities were calculated using Jaccard's similarity coefficient.

The ability of a primer to distinguish large number of genotypes i.e. Resolving Power of the primer (Rp) = $\Sigma\ I_b$. Where, I_b is band informativeness = $1\text{-}|2 \times (0.5\text{-}p)|$ [11], where p being the proportion of the 50 genotypes possessing the band. The Rp of 46 RAPD

primers were determined in this way. The basic information about molecular markers that determines their application in genetic mapping was calculated for each marker using Polymorphism Information Content (PIC). PIC expresses the discriminating power of the locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus, expressed as: PIC = $1 - \Sigma\ pi^2$, where pi is the frequency of the ith (presence of band) allele [12].

Results and discussion

PCR based markers have been used to characterize a wide range of plant species, however, no such reports are available with *Z. mauritiana*, *Z. nummularia* and *Z. spina - christi*. In the present report, RAPD primers were used to study the variation in cultivars of *Z. mauritiana* and their relationship within themselves and also with *Z. nummularia* and *Z. spina-christi*. Genomic DNA samples extracted from different trees of the same varieties demonstrated the reproducibility of the RAPD technique. This contrasts with the reports demonstrating low level of reproducibility of RAPD analysis [13]. The present study suggests that selection of the primers and strict standardization of the protocol are crucial for reproducibility of results.

RAPD markers revealed considerable genetic diversity of *Ziziphus* species with genetic similarity ranging from 26.3% (between Chhuahara and Thornless) to 78.9%. (Narma and Vilayati). Nineteen primers produced genotype specific bands that would prove to be highly useful in identification of genotype and designing future breeding strategy. Thirteen out of these 19 primers were specific for *Z. nummularia* (collection 1) and/or *Z. spina-christi* (Table 2). While four primers *viz.*, OPF-7, OPD-4, OPE-20 and OPF-13 has produced unique bands for Kala Gola, Thornless, Sua and Rohtak Safeda, respectively (Table 2). Two other primers, OPE-11 and OPE-13 amplified unique bands for Tikadi. These nineteen primers have also contributed significantly for high level of polymorphism (86.2%) in the fifty genotypes (Table 2). The results suggest that RAPDs are powerful markers and can be used for varietal identification and purification. Detail information about primers used, total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), resolving power (Rp), polymorphism information content (PIC) and size range of bands obtained are summarized in Table 2.

The resolving power (Rp) was calculated for all the 46 RAPD primers which ranged from 0.48 (OPE-4) to 9.37 (OPD-3) with an average of 3.20 per primer. Besides OPD-3 (9.37), the other RAPD primers *viz.*, OPC-15, OPF-8, OPD-11 and OPE-6 also posses high Rp values of 6.08, 6.01, 5.76 and 5.68, respectively (Table 2). The primers with high Rp values were able to distinguish more number of *ber* genotypes. Polymorphism information content (PIC) was also calculated and it ranged from 0.12 (OPD-20) to 0.82 (OPE-9) with an average of 0.499 per primer. The primers with high PIC values OPE-13 (0.73), OPC-15 (0.72), OPF-2 (0.71) and OPF-8 (0.71) are presented in Table 2. The primers with high Rp and PIC values are more suitable for diversity analysis and fingerprinting of the *ber* genotypes. Graphical relationship between Rp (number of genotypes resolved by individual primer) and PIC (number of alleles analyzed per reaction) values of the primers (Fig. 1) showed linear relationship. The results are in accordance to Huang *et al.* [14] but in contrast to Prasad *et al.* [15] based on their study on wheat.

RAPD profiles : The 46 chosen primers generated a total of 368 fragments (316 polymorphic). Out of 46 polymorphic primers identified, eighteen primers showed 100% polymorphism (Table 2), whereas, the minimum level of polymorphism was shown by OPD-20 (42.86%) with average of 86.2% per primer indicating high marker index. The similar range of polymorphism has been reported by Singh *et al.* [16] based on their study using AFLP. The number of amplification product per primer ranged from 2 (OPF-9) to 17 (OPD-3) with an average of 8 bands per primer (Table 2). A representative DNA profile is presented in figure 2a and 2b.

Genetic relationship based on RAPD markers : The determination of genetic relationships between genotypes is important for *Ziziphus* improvement because till date no effort has been made in this direction. Initial selection and development of varieties has been done with vegetatively propagated material from one orchard to other and from one region to another. The establishment of volunteer seedlings from fallen fruits, consequent selection under differing environments and the lack of qualitative morphological markers has made confusion regarding varietal identity and trueness-to-type. These genetic variants can confound the evaluation of environmental and genetic factors in *ber* research and also can have serious economic ramification for *ber* growers. The taxonomic identity of the common cultivated types of *ber* is confusing [17] and the widely cultivated varieties are very similar with each other [18]. As a consequence, it is difficult to separate them merely on botanical informations. Mixing of the two phenotypically similar genotypes occurs during propagation/nursery stage and disposal of the samples.

The UPGMA dendrogram obtained from the cluster analysis using Jaccard similarity coefficient showed four

Table 2. List of RAPD primers used in the study and their properties on *ber* genotypes

Sl No.	Primer designation	Sequence	TNB*	PNB	P%	Genotype distinguished	Rp	PIC	Size range (bp)
1	OPC-2	5'-GTGAGGCGTC-3'	8	4	50.00	-	1.20	0.27	200-700
2	OPC-3	5'-GGGGGTCTTT-3'	9	7	77.78	-	3.40	0.54	200-900
3	OPC-4	5'-CCGCATCTAC-3'	6	4	66.67	-	2.96	0.45	180-350
4	OPC-6	5'-GAACGGACTC-3'	8	7	87.50	-	3.76	0.51	190-800
5	OPC-7	5'-GTCCCGACGA-3'	7	7	100.00	-	2.52	0.42	500-1500
6	OPC-8	5'-TGGACCGGTG-3'	7	6	85.71	-	3.24	0.48	250-1500
7	OPC-9	5'-CTCACCGTCC-3'	3	3	100.00	-	0.68	0.41	700-1500
8	OPC-10	5'-TGTCTGGGTG-3'	6	5	83.33	*Z. nummularia,Z. spina-christi*	2.24	0.35	450-2000
9	OPC-11	5'-AAAGCTGCGG-3'	10	6	60.00	*Z. nummularia* (2), *Z. spina-christi*	1.44	0.29	450-2000
10	OPC-13	5'-AAGCCTCGTC-3'	5	5	100.00	-	2.88	0.56	500-3000
11	OPC-14	5'-TGCGTGCTTG-3'	6	6	100.00	-	1.52	0.53	500-1000
12	OPC-15	5'-GACGGATCAG-3'	11	11	100.00	*Z. spina-christi*	6.08	0.72	250-1500
13	OPC-16	5'-CACACTCCAG-3'	8	6	75.00	*Z. nummularia* (2)	2.92	0.39	250-1500
14	OPC-18	5'-TGAGTGGGTG-3'	10	8	80.00	*Z. nummularia*	3.64	0.37	150-750
15	OPC-20	5'-ACTTCGCCAC-3'	6	5	83.33	-	2.04	0.49	500-800
16	OPD-3	5'-GTCGCCGTCA-3'	17	16	94.12	-	9.37	0.68	500-5000
17	OPD-4	5'-TCTGGTGAGG-3'	10	9	90.00	Thornless	4.60	0.64	450-1500
18	OPD-7	5'-TTGGCACGGG-3'	14	9	64.29	-	3.51	0.33	350-3000
19	OPD-8	5'-GTGTGCCCCA-3'	7	7	100.00	-	3.24	0.56	450-1100
20	OPD-11	5'-AGCGCCATTG-3'	12	12	100.00	-	5.76	0.53	250-1500
21	OPD-12	5'-CACCGTATCC-3'	6	6	100.00	-	2.08	0.61	550-3000
22	OPD-20	5'-ACCCGGTCAC-3'	7	3	42.86	-	1.00	0.12	200-750
23	OPE-2	5'-GGTGCGGGAA-3'	10	8	80.00	-	3.32	0.53	400-3000
24	OPE-4	5'-GTGACATGCC-3'	6	4	66.67	*Z. nummularia*	0.48	0.38	500-1800
25	OPE-6	5'-AAGACCCCTC-3'	9	9	100.00	-	5.68	0.59	300-3000
26	OPE-7	5'-AGATGCAGCC-3'	3	2	66.67	-	0.92	0.25	450-750
27	OPE-9	5'-CTTCACCCGA-3'	4	4	100.00	-	2.80	0.82	400-1031
28	OPE-11	5'-GAGTCTCAGG-3'	5	3	60.00	Tikadi	2.04	0.46	400-1250
29	OPE-13	5'-CCCGATTCGG-3'	8	7	100.00	Tikadi	3.80	0.73	600-2000
30	OPE-14	5'-TGCGGCTGAG-3'	8	6	75.00	-	3.16	0.63	200-1200
31	OPE-19	5'-ACGGCGTATG-3'	8	6	75.00	-	4.40	0.49	400-1800
32	OPE-20	5'-AACGGTGACC-3'	8	8	100.00	Sua	3.88	0.44	300-1500
33	OPF-1	5'-ACGGATCCTG-3'	11	8	72.73	*Z. nummularia* (3)	4.81	0.42	400-3000
34	OPF-2	5'-GAGGATCCCT-3'	14	13	92.85	-	4.12	0.71	250-3000
35	OPF-3	5'-CCTGATCACC-3'	9	8	88.89	*Z. nummularia*	4.20	0.49	500-3000
36	OPF-4	5'-GGTGATCAGG-3'	7	7	100.00	*Z. nummularia,Z. spina-christi*	1.64	0.35	600-1400
37	OPF-6	5'-GGGAATTCGG-3'	7	5	71.43	-	3.72	0.61	400-3000
38	OPF-7	5'-CCGATATCCC-3'	6	6	100.00	Kala Gola	2.52	0.58	900-3000
39	OPF-8	5'-GGGATATCGG-3'	11	11	100.00	-	6.01	0.71	200-3000
40	OPF-9	5'-CCAAGCTTCC-3'	2	2	100.00	-	1.00	0.41	500-900
41	OPF-10	5'-GGAAGCTTGG-3'	6	5	83.33	-	2.20	0.56	600-2000
42	OPF-11	5'-TTGGTACCCC-3'	12	10	83.33	*Z. nummularia,Z. spina-christi*	4.20	0.62	400-1250
43	OPF-12	5'-ACGGTACCAG-3'	9	8	88.89	*Z. spina-christi*	2.48	0.55	400-2000
44	OPF-13	5'-GGCTGCAGAA-3'	5	4	80.00	Rohtak Safeda	1.76	0.29	400-2000
45	OPF-15	5'-CCAGTACTCC-3'	10	10	100.00	*Z. nummularia*	4.24	0.64	600-3000
46	OPF-16	5'-GGAGTACTGG-3'	7	7	100.00	*Z. spina-christi*	3.92	0.48	400-2000
	Total		368	316	-		-	-	
	Average		-	7.83	86.20		3.20	0.499	

*TNB = Total number of bands; PNB = Number of polymorphic bands; P% = Polymorphism percentage; Rp = Resolving power; PIC = Polymorphic information content.

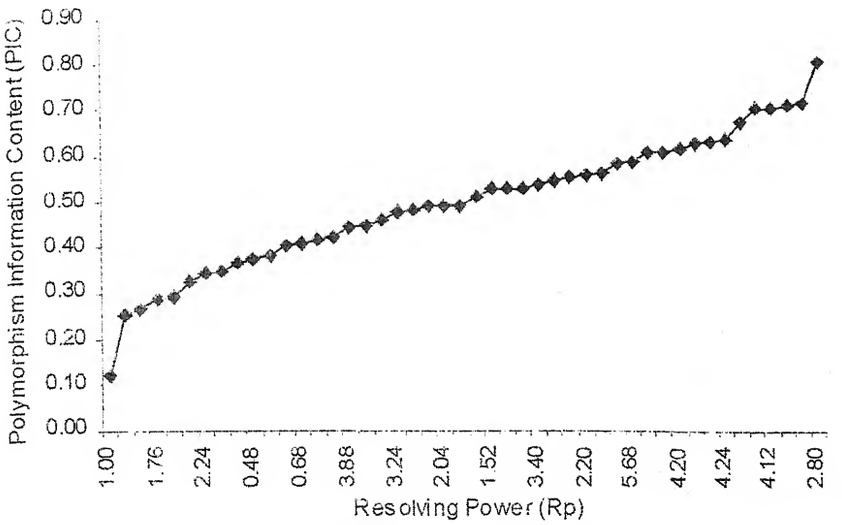
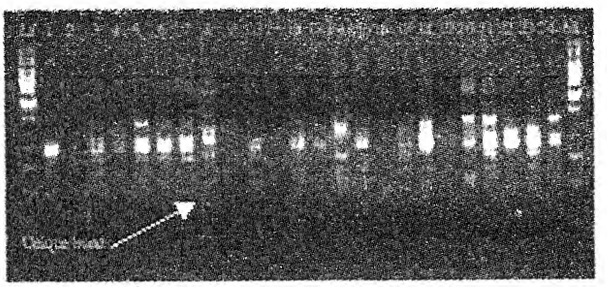
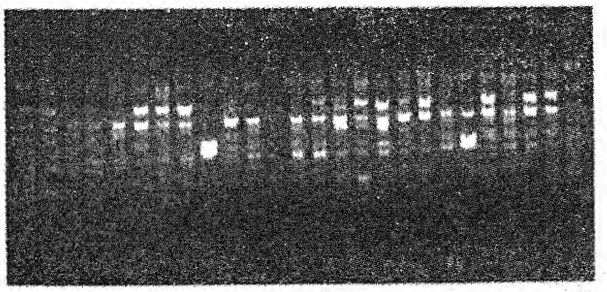


Fig. 1. Graphical relationship between resolving power and polymorphism information content



a: OPD-4 (1-24)



b: OPF-3 (1-24)

Fig. 2. RAPD profile of *ber* accessions generated by primer OPD-4 and OPF-3 (M = 1kbp DNA ladder from MBI Fermentas, 1-24 = *ber* genotypes)

major and two minor clusters (Fig. 3), while *Ziziphus nummularia* collection-1 and *Z. spinachristi* does not fall in any cluster. The cluster-I and cluster-II, both comprised of two genotypes, Seb and Chhuahara and Thronless and Tikadi, respectively. The major clusters (Cluster III to Cluster VI) comprised of remaining 44 genotypes. In cluster-III six genotypes, Rohtak Safeda, Sanauri-3, Bagwadi, Chonchal, Sindhura Narwal and Sanauri were grouped. In cluster IV eleven genotypes (Umran, Tass Bataso, Ilaichi, No-A, Noki, Hesang Tsaon, Jogia, Akhrota, Katha Bombay, Dandan and Gola), in cluster-V, seven genotypes (Ilaichi Jhazzar, Zg-3, Kheera, Kishmish, Nazuk, Reshmi and Pathan), whereas, in the biggest cluster VI, 20 genotypes (Katha Rajasthan, Sua, Popular Gola, *Z. nummularia* collection 2, Pownda, Katha, Muria Mehrara, Bawal Sel-2, Lakhan, Kala Gola, Bawal Sel-1, wild collection of *Z. mauritiana*, Kathi, Govindgarh Sel., Kaithali, Desi Alwar, Narma, Banarasi Karaka and Vilayati) grouped together. The two *Z. nummularia* genotypes were collected from different geographical regions, collection-1 was from Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi, whereas, collection-2 was from Uttar Pradesh, this may be apparent reason of their different grouping. The analysis of grouping pattern for all other genotypes did not correspond to their geographical regions. For instance, cultivars from Rajsthan, Punjab, Uttar Pradesh and Haryana remained together, e.g. Sindhura (from Haryana), Banarasi Karaka (from Uttar Pradesh), Narma (from Delhi) and Desi Alwar (from Rajasthan) grouped in cluster-VI. In contrast the genotypes from the same geographical region were grouped into different clusters e.g. Rohtak Safeda (cluster III) and Kaithali (cluster V) are both from Haryana. This may be explained by the fact that human interventions have played a major role in distribution of *ber* followed by cross-pollination between local and introduced materials. The groupings of samples in one cluster collected from different sub-zones have been reported in neem by Deshwal *et al.* [19].

Our study revealed that the phenotypically similar genotypes could be reliably distinguished using RAPD markers. Morphologically similar genotypes; Katha, Katha Bombay, Katha Rajasthan and Umaran were clustered into two different clusters Katha and Katha Rajasthan (cluster VI) and Katha Bombay and Umraan (cluster IV) and shared 65.03% and 74.72%, respectively. That can be one of the possible reasons for high morphological and flavonoid pattern similarity between these genotypes [1]. The results are in accordance to the suggestions made by Chadha *et al.* [18]. Moreover,

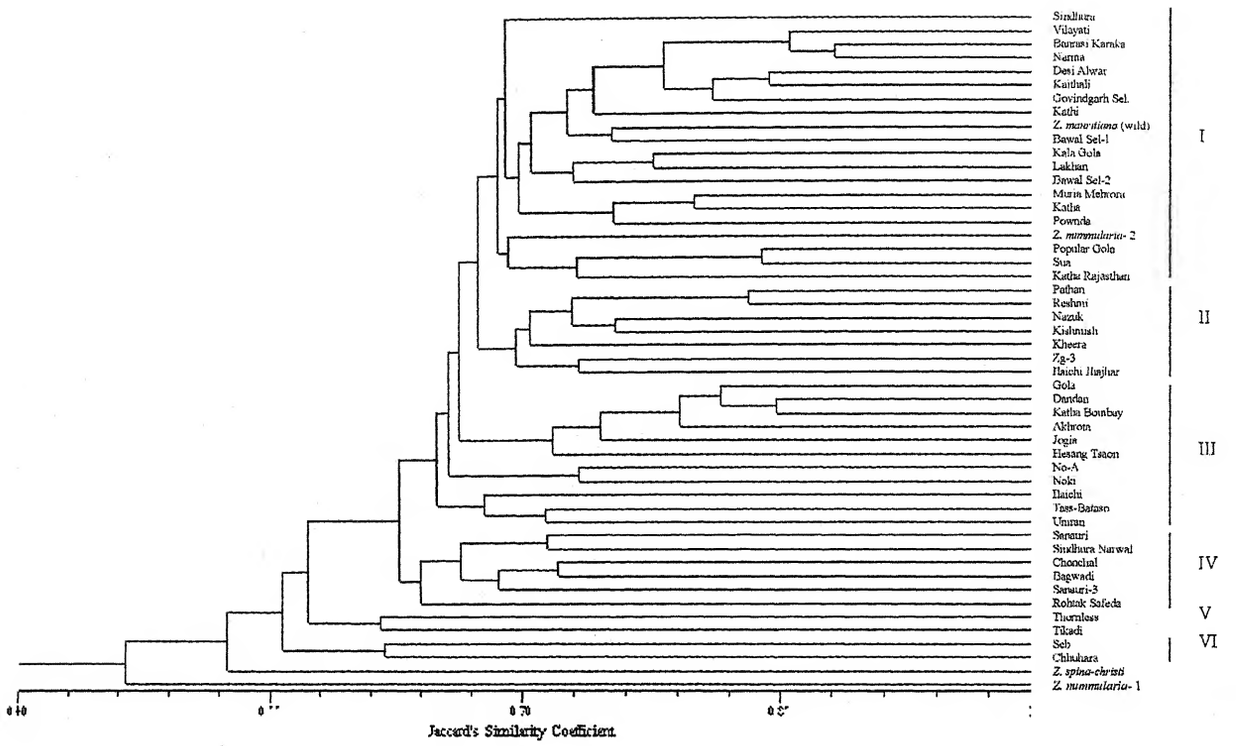


Fig. 3. Genetic relationships among 50 *Ziziphus* genotypes based on Jaccard's smilarity

all the primers except OPC-2, OPC-8, OPC-13, OPC-20, OPE-4 and OPF-7 amplified at least one different band to distinguish the four genotypes namely, Katha, Katha Bombay, Katha Rajasthan and Umran. The genotypes Gola, Akhrota, Seb and Nazuk were reported to be different morphologically, but in the present study Gola and Akhrota grouped together in subcluster IV with 79.39% genetic similarity, whereas, Seb and Nazuk were grouped with the genotypes in subclusters I and V, respectively. Genotype, Seb showed 61.86% genetic similarity with the genotype Chhuhara in the cluster-I. The four genotypes, namely Gola, Nazuk, Akhrota and Seb were distinguishable with all the RAPD primers tested except OPC-4, OPC-13, OPC-16, OPE-4 and OPF-4. The genotype Popular Gola that is morphologically similar to Gola (meaning round in shape) fell into different subclusters IV and VI, respectively. These two genotypes were distinguishable with all the RAPD primers tested except OPC-10, OPE-4, OPE-7, OPE-11 and OPF-12. The result suggests that RAPD markers are able to differentiate and fingerprint different genotypes that are phenotypically similar. The present study has revealed a broad genetic basis in *Ziziphus species* and also suggests that breeding programme can be assisted with the RAPD markers for purification and the identification of a particular genotype.

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